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β-Phenylpyruvate and Glucose Uptake in Isolated Mouse Soleus Muscle and Cultured C2C12 Muscle Cells

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Abstract Previous investigation demonstrated the potential of β-phenylpyruvate at high concentration to cause hypoglycemia in mice totally deprived of insulin. For further elucidation of the glucose-lowering mechanism, glucose uptake, and quantity of glucose transporters (GLUT1 and GLUT4) in mouse soleus muscle and C2C12 muscle cell lines were investigated following incubation with β-phenylpyruvate in various concentrations. A marked enhancement of glucose uptake was demonstrated that peaked at 0.5 and 1.0 mM β-phenylpyruvate in soleus muscle ($P < 0.01$) and C2C12 cells ($P < 0.001$), respectively. Kinetic analysis in C2C12 cells showed a twofold increase in V_{max} compared with controls ($P < 0.001$). In addition, both GLUT1 and GLUT4 levels were increased following exposure to β-phenylpyruvate. Our findings point to a peripheral hypoglycemic effect of β-phenylpyruvate. J. Cell. Biochem. 90: 957–963, 2003.

Key words: phenylpyruvic acid; glucose; skeletal muscle; cultured cells; phenylketonurias; glucose transporters

β-Phenylpyruvate is a natural metabolite of the amino acid phenylalanine and appears in high concentrations in serum of phenylketonuric patients [Levy, 1999]. Two decades ago Sener et al. [1983] showed, in an in vitro model of isolated pancreatic islet cells, that the naturally occurring phenylalanine metabolite 3-phenylpyruvate is an insulin secretagogue. In more recent studies, this group showed that other pyruvate derivatives, such as methyl pyruvate [Jijakli et al., 1996] and *N*-[(*trans*-4-isopropylcyclohexyl)-carbonyl]-D-phenylalanine (A-4166) [Malaisse and Sener, 1998], are also capable of insulin release, as proved in a similar experimental model. We have corroborated these findings and demonstrated, in neonatal mice, that β-phenylpyruvate induces a significant reduction of blood glucose level associated with histological central neural damage attributable to hypoglycemia [Gazit and Katz, 1997].

Reduction of blood glucose can occur via increased insulin release, increased utilization of glucose in peripheral tissues, or decreased gluconeogenesis. As the exact mechanisms by which phenylpyruvate induces its glucose-lowering effect in vivo are still unsettled, we conducted the present study in which we concentrated on the potential of β-phenylpyruvate to decrease blood glucose levels by direct and insulin-unrelated peripheral mechanisms. We investigated this issue by means of an ex vivo model of whole mouse soleus muscle (characterized by its high glucose utilization), at the cellular level using C2C12 cultured muscle cells and at the molecular level by quantifying

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glucose transporters (GLUTs) [Mueckler et al., 1985; Charron and Kahn, 1990].

MATERIALS AND METHODS

Animals

The study was approved by the Animal Use and Care Committee of the Technion Faculty of Medicine. Animals were cared for in accordance with national and institutional guidelines. ICR mice 28 days old were anesthetized with intravenous ketamine (5–7 mg/100 g body weight). The soleus muscles were carefully dissected. Fresh muscle specimens were used for the ex vivo experiment. Other specimens were freeze-dried at −20°C and then stored at −70°C before use.

Glucose Uptake in Isolated Soleus Muscle

Fresh soleus muscles weighing approximately 12 mg were put in separate plates and incubated at 37°C for 2 h in 2 ml glucose-free Krebs–Ringer bicarbonate (KRB) solution, pH 7.4, with the following composition: 119 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, and 1.2 mM $CaCl_2 \cdot 2H_2O$. Plates were bubbled with a gaseous composition of 95% O_2–5% CO_2 at 37°C. After the 2-h glucose-deprivation period, β-phenylpyruvate (Sigma, Petan–Tikva, Israel) in ascending concentrations (0.1–20.0 mM) was added to the incubation solution for 30 min in the experimental group, while muscle specimens in KRB served as control.

To measure uptake of glucose, with or without β-phenylpyruvate, we used the non-metabolized glucose analogue 2-deoxy-D-[2,6-^{3}H]glucose ([^{3}H]2-DG), which is useful for kinetic analysis of glucose transport [Walum and Edström, 1976]. Preliminary experiments on the time course resulted in saturation of glucose uptake 20 min after incubation with [^{3}H]2-DG. As 50% of maximal glucose uptake was calculated to occur at 7 min, this time point was used for further experiments. After incubation with a mixture of [^{3}H]2-DG (1 μCi/ml) and non-radioactive 2-DG (final concentration 5 mM) for 7 min at 37°C, the solution was removed by suction and rapidly washed three times with ice-cold KRB. Soleus muscles from each sample were put in Eppendorf tubes with 0.4 ml of 0.1% SDS and incubated for 2 h at 50°C. In this process, pure muscle cells were separated from fascia and connective tissue [Walum and Edström, 1976]. The extract (containing only myocytes) was counted for radioactivity in 4 ml of Opti-Fluor (Packard Instrument Co., Meridian, CT). Quench correction were used for calculation of dpm.

Non-specific uptake was measured by incubating the muscles with cytochalasin B (40 μM/ml) (Sigma), which binds to GLUTs and inhibits glucose transport into the cell [Jarvis et al., 1986]. Non-specific uptake was subtracted from total uptake to obtain values for specific uptake.

Glucose Uptake in C2C12 Cultured Muscle Cells

C2C12 muscle cells (10^5 cells/mm^2) were grown in 2 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% (v/v) antibiotic solution (50 U/ml penicillin G and 50 U/ml streptomycin) at 37°C in a 5% CO_2-humidified atmosphere until subconfluent density was achieved. After culturing 48 h, cells were transferred to low-glucose (1 g/L) serum-free DMEM for 2 h. Cells were then washed with 1 ml Krebs–Ringer phosphate (KRP) containing 130 mM NaCl, 5.0 mM KCl, 1.3 mM $CaCl_2 \cdot 2H_2O$, 1.3 mM $MgSO_4 \cdot 7H_2O$, and 10.0 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4. To determine the effect of β-phenylpyruvate on glucose uptake, 30 min incubation in 1 ml of KRP with or without increasing concentrations (0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mM) of β-phenylpyruvate was carried out. The reaction was performed by adding a mixture of [^{3}H]2-DG (1 μCi/ml) and non-radioactive 2-DG (final concentration 5 mM) for 3 min. The solution was removed by suction and the cells rapidly washed three times with ice-cold KRP. C2C12 muscle cells from each sample were put in Eppendorf tubes with 0.4 ml of 0.1% SDS. The extract was counted for radioactivity in 4 ml of Opti-Fluor. Quench correction were used for calculation of dpm.

Non-specific uptake was measured by incubating the cells with cytochalasin B (40 μM/ml). Non-specific uptake was subtracted from total uptake to obtain values for specific uptake. Results were expressed as pmol/3 min/10^5 cells.

Kinetics of Glucose Uptake

The kinetics of glucose transport is based on facilitated diffusion [Stein et al., 1974; Mueckler et al., 1985] and can be analyzed similarly to an enzymatic reaction [Bradford, 1976; Zivin and Waud, 1982]. The rate of glucose uptake

in the C2C12 cells in the presence or absence of β-phenylpyruvate (1.0 mM) was calculated. The Michaelis–Menten constant (K_m) and maximum transport velocity (V_{max}) were computed using Lineweaver–Burk analysis [Zivin and Waud, 1982].

In order to exclude a possible direct cytotoxic effect of β-phenylpyruvate on C2C12 muscle cells, we used Alamar Blue (Biosource International, Camarillo, CA). This indicator is used to measure cytotoxicity of various chemicals in cultured cells. The use of Alamar Blue is based on the ability of cellular enzymes to reduce the non-fluorescent blue dye to a pink fluorescent compound, allowing detection of cell viability by spectrophotometry [Back et al., 1999]. Alamar Blue (10% v/v) was added to the C2C12 cell culture for 30 min at 37°C. Cytotoxicity was determined by measuring absorbance at a wavelength of 570 nm and subtracting background absorbance measured at 600 nm.

β-Phenylpyruvate and GLUTs in Soleus Muscle and C2C12 Cells

Fresh tissue samples of soleus muscle were washed and C2C12 muscle cell lines were scraped from the plates and put into a lysis buffer containing 1% Triton X-100, 0.1% SDS, and one tablet of α-anti-proteinase inhibitor in PBS, pH 7.4, prior to light homogenization at 4°C. Lysates were centrifuged at 10,000*g* for 10 min at 4°C and the supernatant was used for Western analysis of GLUTS in cytosol and microsomal fractions.

To assess the isoform-specific subcellular quantities of GLUT1 and GLUT4, membrane samples (30 μg protein) of soleus muscles incubated with different concentrations (0.1–10 mM) of β-phenylpyruvate for 7 min at 37°C (see "Glucose Uptake in Isolated Soleus Muscle") and C2C12 cells incubated for 30 min at 37°C in 5% CO_2-humidified atmosphere (see "Glucose Uptake in C2C12 Cultured Muscle Cells") were diluted in Laemmli buffer (2% SDS, 65 mM Tris, 8% glycerol, and 0.04% bromophenol blue) (Bio-Rad, Hercules, CA) with mercaptoethanol and electrophoretically resolved on graded 4–12% SDS–PAGE duplicate gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad) using semidry electroblotter (Invitrogen, Carlsbad, CA). The membranes were saturated with blocking buffer (5% non-fat dry milk, 0.1% Tween-20, 0.5% BSA, and 0.5 M NaCl in 50 mM Tris, pH 7.5) (Bio-Rad) for 1 h at room temperature. Each of the duplicate membranes was then incubated overnight at 4°C in a solution containing either rabbit anti-GLUT1 or anti-GLUT4 polyclonal antiserum (Chemicon International, Temecula, CA) diluted 1:5,000 in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, and 0.1% Tween-20) containing 1% (w/v) BSA. After exposure to the primary antiserum, the membranes were washed three times for 15 min each at room temperature in TBST and then incubated for 1 h with goat anti-rabbit IgG-horseradish peroxidase (1:10,000) (Sigma). Antibody binding was detected by enhanced chemiluminescence using AmpLight™ Chemi-Luminescent Western Detection kit (Bio-Rad) and quantified by densitometry. Insulin (100 nM, Sigma) was used as a positive control for glucose uptake.

Statistical Analysis

All values are given as means ± SEM. Differences among groups were analyzed using repeated-measures ANOVA, and post-hoc analysis was performed by the Newman–Keuls multiple comparison tests.

RESULTS

Glucose Uptake in Soleus Muscle

Glucose uptake was measured by adding [^{3}H]2-DG. Saturation of glucose uptake occurred 20 min after the addition of [^{3}H]2-DG. Fifty percent of maximal glucose uptake was calculated from the saturation curves to occur 7 min following the incubation with [^{3}H]2-DG. This time point was used for the other experiments.

Figure 1 shows that exposure to β-phenylpyruvate at increasing concentrations of 0.1–20 mM produced an increase (peaking at 5 mM) followed by a decrease in glucose uptake as compared with control. It could be that exposure to higher concentration of β-phenylpyruvate (>7 mM) caused a direct myotoxic effect leading to the decrease in glucose uptake observed in the isolated soleus muscle.

Glucose Uptake in C2C12 Muscle Cells

Saturation of glucose uptake occurred 6 min after the addition of [^{3}H]2-DG. Fifty percent of maximal glucose uptake was calculated to occur 3 min following the incubation with [^{3}H]2-DG. This time point was used for the other experiments.

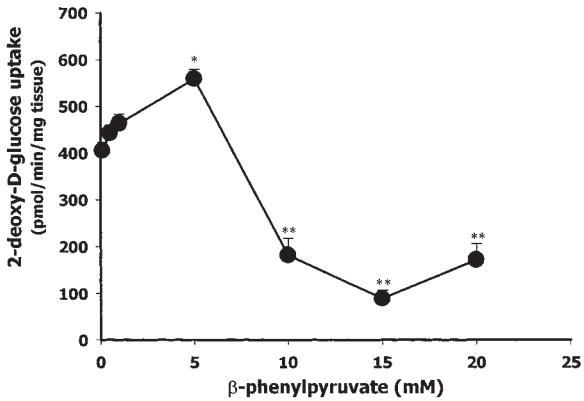

Fig. 1. Effect of β-phenylpyruvate on 2-deoxy-D-[2,6-^{3}H]glucose ([^{3}H]2-DG) uptake in soleus muscle ($n = 6$). Soleus muscles were incubated at 37°C for 2 h in glucose-free KRB solution. β-Phenylpyruvate at various concentrations (0.1–20 mM) was added for 30 min. Glucose uptake (pmol/min/mg tissue) was measured 7 min following exposure to [^{3}H]2-DG. *, $P < 0.01$; **, $P < 0.001$.

Figure 2 demonstrates the effect of different concentrations of β-phenylpyruvate on glucose uptake into C2C12 muscle cells. The uptake of [^{3}H]2-DG increased 3.3-fold at a concentration of 1.0 mM β-phenylpyruvate ($n = 6$; $P < 0.001$) during the 3 min following incubation with β-phenylpyruvate.

Alamar Blue

Incubation of Alamar Blue with C2C12 muscle cells together with β-phenylpyruvate (0.1–5.0 mM) produced a significant reduction of 20 and 30% of C2C12 muscle cell absorbance over the 4-h incubation period at concentrations of 2.0 and 5.0 mM β-phenylpyruvate, respectively (data not shown). The cytotoxic effect of β-phenylpyruvate was taken in consideration when glucose uptake in cells was calculated.

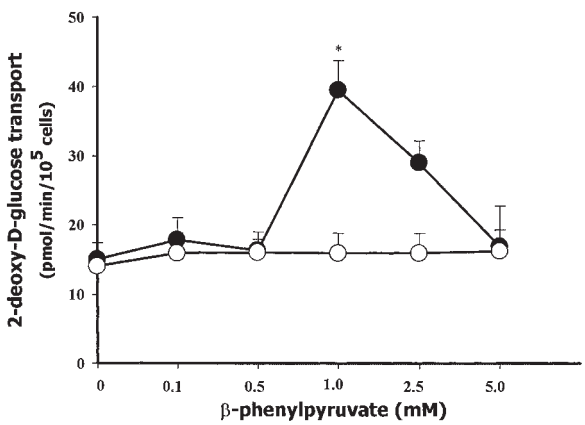

Fig. 2. Effect of β-phenylpyruvate on [^{3}H]2-DG uptake in C2C12 muscle cells ($n = 6$). C2C12 cells were incubated at 37°C for 2 h in low-glucose, serum-free DMEM. Krebs–Ringer phosphate (KRP) with β-phenylpyruvate at various concentrations (0.1–5 mM) or without (control) was added 30 min before the end of incubation. Glucose uptake (pmol/min/10^5 cells) was measured 3 min following exposure to [^{3}H]2-DG. *, $P < 0.001$; ●, β-phenylpyruvate.

Kinetics of [^{3}H]2-DG Uptake in C2C12 Cells

Using the constructed Lineweaver–Burk curve, the calculated V_{max} value for [^{3}H]2-DG uptake was found to be significantly higher than in control (2.7 ± 0.15 to 6.1 ± 0.3 pmol/3 min/10^5 cells vs. 1.6 ± 0.3 to 2.2 ± 0.15 pmol/3 min/10^5 cells), without changing the K_m values (Fig. 3). The observed increase in V_{max} appeared to result from translocation of existing glucose carriers from intracellular sources to the cell surface.

Effect of β-Phenylpyruvate on GLUT1 and GLUT4 Levels in Soleus Muscle and C2C12 Muscle Cells

The expression of GLUT1 and GLUT4 proteins was investigated by immunoblotting in soleus muscle cells (Fig. 4) and C2C12 muscle cells (Fig. 5). It can be seen that both GLUT1 and GLUT4 levels were increased (tenfold and twofold, respectively) in soleus muscle exposed to 1 mM β-phenylpyruvate as compared with control ($n = 6$; $P < 0.001$). Similarly, β-phenylpyruvate at a concentration of 10 mM increased both GLUT1 and GLUT4 levels (fourfold and twofold, respectively) as compared with control ($n = 61\ P < 0.01$). Figure 5 demonstrates that in C2C12 muscle cells, GLUT4 increased 3.3- or 3.0-fold as compared with control when cells

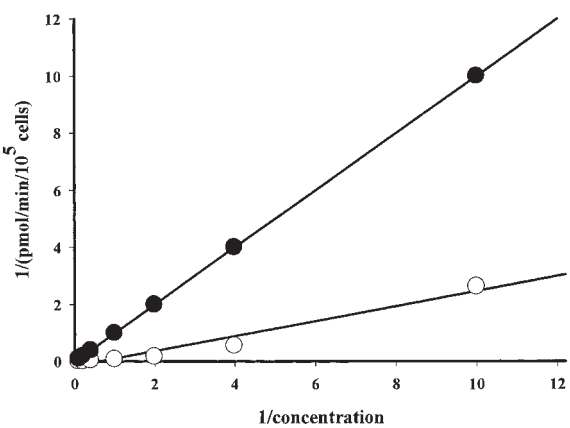

Fig. 3. Kinetics of [^{3}H]2-DG transport by C2C12 muscle cells. Using the constructed Lineweaver–Burk curve, the calculated V_{max} value for [^{3}H]2-DG uptake was found to be significantly higher than in the control group, without changing the K_m values. $n = 6$; $P < 0.001$; ●, β-phenylpyruvate (1 mM); ○, control.

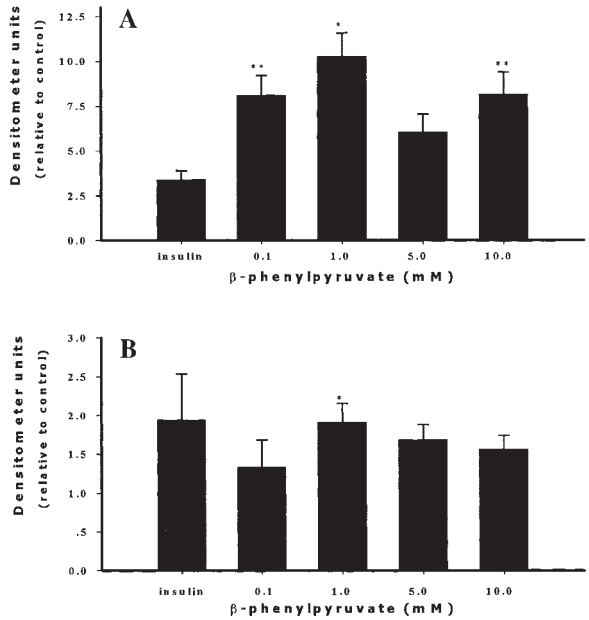

Fig. 4. Quantification of Western blots of GLUT1 (**A**) and GLUT4 (**B**) in soleus muscle plasma membranes. Both GLUT1 and GLUT4 levels were increased following exposure to β-phenylpyruvate for 7 min. *, $P < 0.001$; **, $P < 0.01$ versus no insulin. Insulin (100 nM) was used as a positive control for glucose uptake and cytochalasin B for non-specific binding.

were incubated with β-phenylpyruvate at concentrations of 0.1 and 1.0 mM, respectively.

DISCUSSION

β-Phenylpyruvate is a byproduct of transamination of the amino acid phenylalanine. It is produced in excess when the alternative metabolic pathway for phenylalanine supervenes, as occurs when phenylalanine hydroxylase, which is responsible for normal phenylalanine hydroxylation, is deficient, as in the inherited metabolic disease phenylketonuria [Levy, 1999]. Previous investigation suggested a possible potential of β-phenylpyruvate at high concentrations to cause hypoglycemia [Sener et al., 1983] with associated neural damage in mice [Gazit and Katz, 1997]. We also observed that β-phenylpyruvate retained its hypoglycemic effect after chemical destruction of the islets of Langerhans, hinting at a possible peripheral and insulin-independent mechanism [Gazit and Katz, 1997]. Furthermore Sener et al. [1993], showed a non-glucidic insulin releasing capacity of phenylpyruvate analogue in adult rats treated in the neonatal period with streptozotocin suggesting other not completely understood mechanisms of action of this keto-acid in connection with glucose homeostasis.

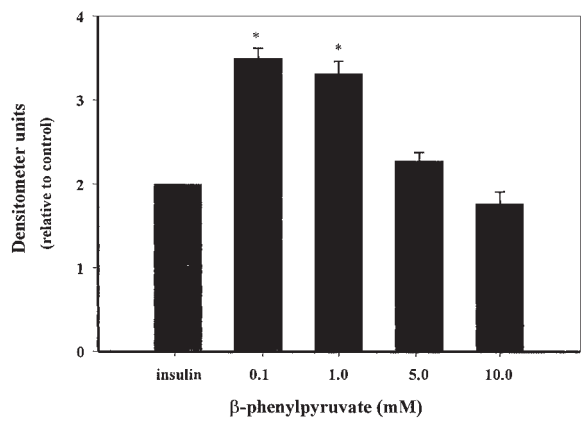

Fig. 5. Quantification of Western blots of GLUT4 in C2C12 muscle cell plasma membranes. GLUT4 levels were increased following exposure to β-phenylpyruvate for 30 min. *, $P < 0.001$ versus no insulin. Insulin (100 nM) was used as a positive control for glucose uptake and cytochalasin B for non-specific binding.

In the present complementary study, we tried to investigate the peripheral effects of β-phenylpyruvate on blood glucose levels using ex vivo, cellular and molecular experimental models. We chose to explore the β-phenylpyruvate-associated effect on facilitative glucose uptake in two different tissue preparations, isolated soleus and C2C12 muscle cell cultures. The soleus muscle preparation was chosen because of its known high-energy consumption and glucose utilization [Tsao et al., 2001]. Similarly, the fusogenic mouse skeletal muscle cell line, C2C12, as a subclone of the C2 cell line, has been used by many researchers because of its high reproducibility and the rapidity with which acute changes can be produced by altering media conditions [Shimokawa et al., 2000].

In the present study, we found that β-phenylpyruvate increased both glucose uptake and quantity of GLUT1 and GLUT4 in isolated soleus muscle and GLUT4 in C2C12 muscle cell culture. The β-phenylpyruvate-associated specific increase in glucose uptake was reflected by higher intracellular transport of its traced analogue [^{3}H]2-DG. Incubation with β-phenylpyruvate up to a concentration of 5.0 mM resulted in a significant increase of measured glucose uptake, whereas exposure to higher concentrations resulted in a decrease in measured glucose uptake, probably due to certain myotoxic effects of β-phenylpyruvate in this higher concentration range [Gazit et al., 1998].

In addition, kinetic analysis of the glucose uptake in the C2C12 muscle cells was associated with higher V_{max} but with the same K_m

value as compared with control (non-β-phenylpyruvate-treated group), reflecting the ability of β-phenylpyruvate to recruit GLUTs from the intracellular space to the cell surface without changing their structure and function. Indeed, GLUT proteins were previously shown to be recruitable [Bryant et al., 2002]. However, the relevance of the findings in C2C12 muscle cells to the whole muscle has still not been determined.

Since the cell is impermeable to glucose, glucose is transported by specific carrier proteins, or transporters, that allow the binding and transfer of glucose across the hydrophobic lipid bilayer. The regulation of glucose transport in adipose, skeletal, and cardiac muscle is mainly a function of two GLUTs. One of these GLUT isoforms, known as the erythrocyte type, or GLUT1, is widely expressed in non-insulin-responsive tissues [Rodnick et al., 1990]. The other transporter isoform, the muscle-adipose type, or GLUT4, is expressed only in those cells where glucose uptake is stimulated by insulin (i.e., myocytes) [Thorens et al., 1990] and is thought to be the major transporter in these tissues [Kahn, 1992]. In rat adipocytes, GLUT4 is localized in the intracellular space, where it is stored in vesicles that can be differentiated and regulated according to various physiological or pathological conditions. For example, a strong decrease in GLUT4 expression, but not of GLUT1, has been reported in adipocytes of diabetic and fasted rats [Charron and Kahn, 1990]. The expression of GLUT4 can be restored by insulin treatment or by refeeding fasted animals [Charron and Kahn, 1990]. Changes in GLUT4 expression in muscle have also been shown in diabetic patients following exercise [Rodnick et al., 1990]. All the above-mentioned data were revealed using in vivo models. Furthermore, most data concerning translocation of GLUTs from intracellular membranes and their attachment to the cell membrane as a function of various stimuli were accumulated from experimental models using fat cells, and less is known about a similar mechanism in skeletal muscle and in myocytes, where insulin plays a significant role. Our study demonstrated that changes in GLUT levels occur in ex vivo models following exposure to β-phenylpyruvate. Interestingly, β-phenylpyruvate seems to mobilize GLUT1 more potently than GLUT4 to the plasma membrane of soleus muscle resulting in an increase in glucose uptake seen in the presence of β-phenylpyruvate.

In this study, we found a significant rise in glucose uptake in isolated soleus muscle and cultured C2C12 muscle cells exposed to β-phenylpyruvate in the absence of insulin. These findings hint at a peripheral mechanism underlying the glucose-lowering effects of β-phenylpyruvate and possibly the β-phenylpyruvate-induced increase in GLUTs. Indeed, previous study in an intact animal demonstrated the ability of GLUT proteins to be upregulated by different agents [Zorzano et al., 1998]. Furthermore, it has been demonstrated that β-phenylpyruvate might be involved in the activation of the GLUT protein by its phosphorylation, thus explaining its hypoglycemic effect [Gazit et al., 1998].

Glucose is a primary energy source for human biological systems. The CNS is especially dependent on a continuous supply of glucose, because it cannot store a supply sufficient for more than a few minutes of activity [Boyle et al., 1995]. Accordingly, severe neural damage can occur following a short period of hypoglycemia [Auer, 1998]. Given the fact that β-phenylpyruvate is present in excessive amounts in phenylketonuric patients, it may trigger episodes of hypoglycemia and subsequent neural damage.

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