

Palmitate acutely raises glycogen synthesis in rat soleus muscle by a mechanism that requires its metabolism (Randle cycle)

Sandro Massao Hirabara^a, Carla Roberta de Oliveira Carvalho^a, José Roberto Mendonça^a, Esther Piltcher Haber^a, Luiz Claudio Fernandes^b, Rui Curi^{a,*}

^aDepartment of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, Cidade Universitária, Av. Professor Lineu Prestes 1524, Butantã, São Paulo, SP 05508-900, Brazil

^bDepartment of Physiology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR 81530-900, Brazil

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Abstract The acute effect of palmitate on glucose metabolism in rat skeletal muscle was examined. Soleus muscles from Wistar male rats were incubated in Krebs–Ringer bicarbonate buffer, for 1 h, in the absence or presence of 10 mU/ml insulin and 0, 50 or 100 μ M palmitate. Palmitate increased the insulin-stimulated [¹⁴C]glycogen synthesis, decreased lactate production, and did not alter D-[U-¹⁴C]glucose decarboxylation and 2-deoxy-D-[2,6-³H]glucose uptake. This fatty acid decreased the conversion of pyruvate to lactate and [1-¹⁴C]pyruvate decarboxylation and increased ¹⁴CO₂ produced from [2-¹⁴C]pyruvate. Palmitate reduced insulin-stimulated phosphorylation of insulin receptor substrate-1/2, Akt, and p44/42 mitogen-activated protein kinases. Bromopalmitate, a non-metabolizable analogue of palmitate, reduced [¹⁴C]glycogen synthesis. A strong correlation was found between [U-¹⁴C]palmitate decarboxylation and [¹⁴C]glycogen synthesis ($r=0.99$). Also, palmitate increased intracellular content of glucose 6-phosphate in the presence of insulin. These results led us to postulate that palmitate acutely potentiates insulin-stimulated glycogen synthesis by a mechanism that requires its metabolism (Randle cycle). The inhibitory effect of palmitate on insulin-stimulated protein phosphorylation might play an important role for the development of insulin resistance in conditions of chronic exposure to high levels of fatty acids.

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Key words: Palmitate; Soleus muscle; Glucose metabolism; Glycogen synthesis; Insulin signalling

1. Introduction

Increased plasma levels of free fatty acids (FFA) occur in states of insulin resistance such as type 2 diabetes mellitus and obesity [1–3]. These high levels of plasma FFA have been postulated to play an important role for the development of

insulin resistance but the mechanisms involved remain to be fully established.

In 1963, Randle et al. [4] described the fatty acid–glucose cycle (the Randle cycle) to explain insulin resistance. These authors showed that FFA decrease glucose utilization by isolated rat heart and diaphragm. The hypothesis is that increased FFA oxidation enhances the generation of acetyl-CoA that leads up to increased citrate production through the Krebs cycle. Citrate, in association with an increase in the ATP/ADP ratio, inhibits phosphofructokinase, resulting in reduced glycolytic flux. As a consequence, there is an increase in the glucose 6-phosphate content, which inhibits hexokinase II activity, leading to an increase in intracellular glucose content and a reduction in glucose uptake [4,5]. However, studies with nuclear magnetic resonance showed that FFA decrease the glucose and glucose 6-phosphate intracellular content [6,7], suggesting that other mechanisms are involved in the FFA-induced insulin resistance.

FFA affect the activity of insulin signalling proteins in different cell lines and mammal tissues: insulin receptor activity, insulin receptor substrate-1 (IRS-1) and protein kinase B (PKB) phosphorylation in pmi 28 cells [8]; insulin receptor affinity [9] and glucose transporter-4 (GLUT-4) intrinsic activity [10] in adipocytes; mitogen-activated protein (MAP) kinase activity in fibroblasts [11]; MAP kinase, phosphatidylinositol 3-kinase (PI3-K) [12], Akt and atypical protein kinase C (PKC) activities [13] in C2C12 myotubes; and IRS-1 phosphorylation, PI3-K activity-associated IRS-1 [14–16], PKB and atypical PKC activities [17,18], and GLUT-4 translocation [17,19] in skeletal muscle. These effects were accompanied by reduced insulin responses such as glucose uptake and glycogen synthesis [8,12–14,17].

Contrary to the information above, other studies suggest that FFA acutely increase insulin action. Palmitate acutely raises both basal and insulin-stimulated glucose uptake in adipocytes [20–22]. Rats subjected to euglycemic-hyperinsulinemic glucose clamp, combined with acute FFA infusion, show a larger whole body glucose utilization and muscle glycogen synthesis [23]. However, if the FFA infusion is longer than 2 h, glucose utilization and glycogen synthesis rapidly decline [24,25]. Thus, the effects of FFA on glucose metabolism and insulin signalling pathways still remain controversial. In this study the acute effects (up to 1 h) of palmitate on glucose metabolism and on insulin signalling pathway that involves phosphorylation of IRS-1/2, Akt, and p44/42 MAP kinases in incubated rat soleus muscle were investigated.

*Corresponding author. Fax: (55)-11-3091 7285.

E-mail address: ruicuri@fisio.icb.usp.br (R. Curi).

Abbreviations: FFA, free fatty acids; GLUT-4, glucose transporter-4; IRS, insulin receptor substrate; MAP kinases, mitogen-activated protein kinases; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B (also known as Akt); PKC, protein kinase C

2. Materials and methods

2.1. Material

All enzymes and reagents for buffers were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Regular insulin was obtained from Biobrás (M. Claros, MG, Brazil). D-[U-¹⁴C]Glucose, 2-deoxy-D-[2,6-³H]glucose, [1-¹⁴C]- and [2-¹⁴C]pyruvate, and ECL Western Blotting System Kit were obtained from Amersham International (Bucks, UK). [U-¹⁴C]Palmitate and L-[1-¹⁴C]glucose were obtained from NEN Life Sciences Products (Boston, MA, USA). Anti-phosphoserine(473)-Akt and anti-phospho-p44/42 (Thr202/Tyr204) MAP kinase antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-phosphotyrosine and anti-IRS-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IgG polyclonal antibody conjugated to horseradish peroxidase was obtained from ICN Biomedicals (Irvine, CA, USA).

2.2. Animals

Male Wistar rats weighing 140–160 g were obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo. Animals were housed under a 12:12 h light–dark cycle, at 23 ± 1°C. The chow was removed 4 h before the beginning of the experiment, which was always carried out at 11.00 h. The study was approved by the Ethical Committee of the Institute of Biomedical Sciences, University of São Paulo.

2.3. Effect of palmitate on glucose metabolism in incubated soleus muscle

Soleus muscles were isolated and incubated as previously described [26,27]. Rats were killed by cervical dislocation and soleus muscles were rapidly and carefully isolated, split longitudinally into two equal portions, weighed (20–30 mg) and pre-incubated for 30 min, at 37°C, in Krebs–Ringer bicarbonate buffer pre-gassed for 30 min with 95% O₂/5% CO₂, containing 5.6 mM glucose, pH 7.4, with agitation at 120 rpm. After this period, the muscles were transferred to other vials containing the same buffer, but supplemented with 0.3 µCi/ml D-[U-¹⁴C]glucose and 0.2 µCi/ml 2-deoxy-D-[2,6-³H]glucose and glucose 5.6 mM. Incubation was then performed for 1 h under similar conditions, in the absence or presence of 10 mU/ml insulin and 0, 50 or 100 µM palmitate (previously dissolved in ethanol, solution of palmitate at 20 mM). A high concentration of insulin was used to pronounce the effect of palmitate. As control, some muscles were incubated in the presence of 0.5% ethanol. Previous experiments had demonstrated that 0.5% ethanol does not alter the responsiveness to insulin (data not shown). Measurements of lactate production from pyruvate (2 mM), [1-¹⁴C]- and [2-¹⁴C]pyruvate decarboxylation (0.2 µCi/ml) were also carried out in muscles incubated for 1 h in the presence of insulin (10 mU/ml) and palmitate (100 µM) and the results were compared with those of insulin only.

Phenylethylamine (0.3 ml solution 1:1 in methanol) was added into a separate compartment to measure ¹⁴CO₂ adsorption. After the incubation period, the muscles were briefly washed in saline at 4°C and frozen in liquid N₂. [¹⁴C]Glycogen synthesis (as estimated by D-[¹⁴C]glucose incorporation into glycogen) was determined as described by Leighton and Cooper [28]. Lactate production was determined as described by Engel and Jones [29]. Decarboxylation of D-[¹⁴C]glucose, [1-¹⁴C]- and [2-¹⁴C]pyruvate and uptake of 2-deoxy-D-[2,6-³H]glucose were measured as previously described [30–32]. For determination of the extracellular space, some muscles were incubated in the presence of 0.1 µCi/ml L-[1-¹⁴C]glucose [33]. Total glycogen content of the muscles was determined as described by Leighton et al. [34].

2.4. Effect of palmitate on insulin signalling proteins

Muscles were incubated for periods of 5, 30 or 60 min in the presence of 100 µM palmitate. Insulin (10 mU/ml) was added to the medium and the muscles incubated for 5 min more. This period of insulin stimulation is sufficient to cause maximal phosphorylation of the proteins studied (data not shown). For comparison, some muscles were incubated for 5 min in the absence (control group) or presence of 10 mU/ml insulin (insulin group) and others for 30 min in the presence of 100 µM palmitate only. At the end of the incubation period, muscles were immediately homogenized in 0.6 ml extraction buffer (100 mM Trizma, pH 7.5; 10 mM EDTA; 10% sodium dodecyl sulfate (SDS); 100 mM NaF; 10 mM sodium pyrophosphate; 10 mM

sodium orthovanadate; at 100°C) for 30 s. Samples were boiled for 5 min and centrifuged at 12 000 rpm, for 40 min, at 4°C. Aliquots of supernatants were used for the measurement of total protein content, as described by Bradford [35]. Equal amounts of proteins of each sample were separated using 6% SDS–polyacrylamide gel [36]. Western blotting was carried out following the method described by Towbin et al. [37]. The proteins of the gel were transferred to a nitrocellulose membrane at 120 V for 1 h. Non-specific bounds were blocked by incubating the membranes with 5% defatted milk in basal solution (10 mM Trizma, pH 7.5; 150 mM NaCl; 0.05% Tween 20) at room temperature, for 2 h. Membranes were washed in basal solution three times for 10 min each and then incubated with anti-phosphotyrosine, anti-IRS-1, anti-phosphoserine(473)-Akt or anti-phospho-p44/42 MAP kinase antibodies in basal solution containing 3% defatted milk, at room temperature, for 3 h. Membranes were washed again (three times for 10 min each) and incubated with anti-IgG antibody linked to horseradish peroxidase in basal solution containing 1% defatted milk, at room temperature, for 1 h. Following washing again, membranes were incubated with substrate for peroxidase and chemiluminescence enhancer (Amersham Pharmacia Biotech) for 1 min and immediately exposed to X-ray film for 1–20 min. Films were then revealed in the conventional manner.

2.5. Effect of bromopalmitate on [¹⁴C]glycogen synthesis in incubated soleus muscle

To verify if the alterations in [¹⁴C]glycogen synthesis by palmitate were due to its metabolization, the non-metabolizable analogue of this fatty acid, bromopalmitate, was tested at 100 µM. Bromopalmitate and palmitate compete equivalently for the same ligand binding sites and tissue-specific transport [38]. Results of *in vivo* studies demonstrate bromopalmitate tracer as a valuable tool for the assessment of tissue-specific total fatty acid uptake [39]. The procedure used was the same as described for palmitate.

2.6. Correlation between [¹⁴C]glycogen synthesis and [U-¹⁴C]palmitate decarboxylation

Muscles were incubated for periods of 15, 30 and 60 min to determine concomitantly both [¹⁴C]glycogen synthesis and [U-¹⁴C]palmitate decarboxylation in the presence of 10 mU/ml insulin and 100 µM palmitate. Determination of [¹⁴C]glycogen synthesis was carried out as described above [28]. To measure [U-¹⁴C]palmitate decarboxylation, the fatty acid was added into the incubation medium at 0.2 µCi/ml. The method to determine [U-¹⁴C]palmitate decarboxylation was the same as utilized for D-[U-¹⁴C]glucose [30].

2.7. Effect of palmitate on intracellular content of glucose 6-phosphate in incubated soleus muscle

Muscles were incubated for 1 h in the absence or presence of 10 mU/ml insulin and/or 100 µM palmitate. The muscles were then frozen in liquid N₂ and the glucose 6-phosphate content was determined as described by Lang and Michal [40].

2.8. Statistical analysis

The data are presented as mean ± S.E.M. and analyzed by one-way ANOVA and Tukey test (*P* < 0.05). Linear regression analysis was used to determine the correlation between [¹⁴C]glycogen synthesis and [U-¹⁴C]palmitate decarboxylation. Band intensities were quantified by optical densitometry using the Scion Image program (Frederick, MD, USA).

3. Results

3.1. Effect of palmitate on glucose metabolism in incubated soleus muscle

Palmitate itself had no effect on basal glycogen synthesis in incubated soleus muscle (Fig. 1A). However, the simultaneous incubation of palmitate at 50 or 100 µM with 10 mU/ml insulin induced a significant increase of glycogen synthesis. The increases were 17% (*P* < 0.05) and 62% (*P* < 0.001) at 50 and 100 µM, respectively (Fig. 1A). Total glycogen content of the muscles was not changed by all treatments imposed. The mean value at the end of 1 h incubation period was

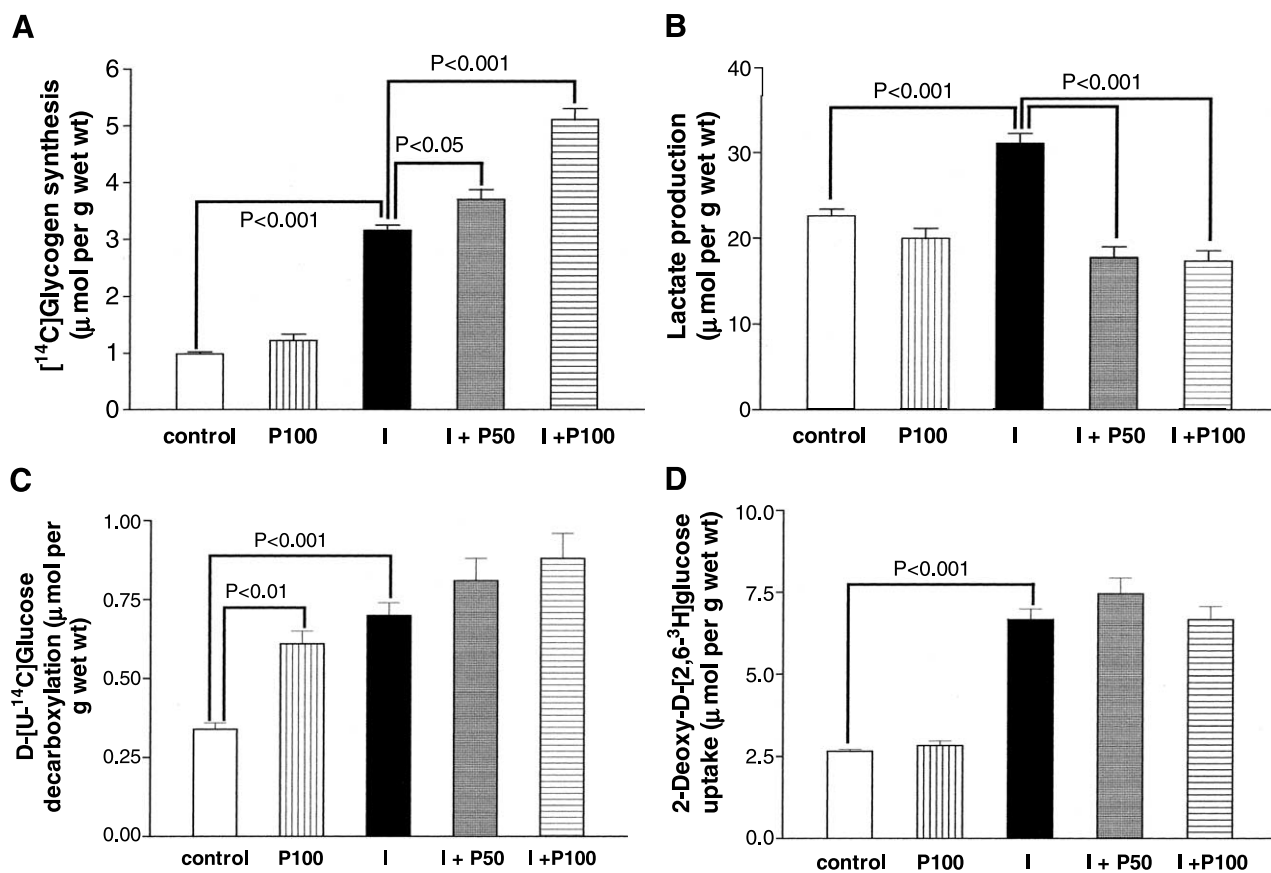


Fig. 1. Effect of palmitate (P) on glucose metabolism in soleus muscle. Muscles were incubated in Krebs–Ringer bicarbonate buffer, containing 5.6 mM glucose, 0.3 μ Ci/ml D-[¹⁴C]glucose, and 0.2 μ Ci/ml 2-deoxy-D-[2,6-³H]glucose, for 1 h in the absence or presence of 10 mU/ml insulin (I) and 0, 50 (P50) or 100 (P100) μ M palmitate. [¹⁴C]Glycogen synthesis (A), lactate production (B), [U-¹⁴C]glucose decarboxylation (C) and 2-deoxy-D-[2,6-³H]glucose uptake (D) were determined as described by Leighton and Cooper [28]; Engel and Jones [29]; Ceddia et al. [30,32], respectively. Data are presented as mean \pm S.E.M. ($n = 24$).

25.96 \pm 0.90, expressed as μ mol per g muscle fresh weight and presented as mean \pm S.E.M. of six determinations from two experiments.

Palmitate 100 μ M had no effect on basal lactate production but it completely abolished the increase of lactate production induced by insulin at both 50 and 100 μ M concentrations ($P < 0.001$) (Fig. 1B). Basal glucose decarboxylation was increased by 100 μ M palmitate (79%; $P < 0.05$) (Fig. 1C). In the presence of 10 mU/ml insulin, this fatty acid led to a slight increase of this parameter (Fig. 1C). Neither basal nor insulin-stimulated 2-deoxy-D-glucose uptake was altered by palmitate (Fig. 1D).

In the presence of insulin, palmitate (100 μ M) caused a significant ($P < 0.05$) decrease of the conversion of pyruvate into lactate (from 10.20 \pm 0.99 to 5.41 \pm 0.50), [¹⁻¹⁴C]pyruvate decarboxylation (from 3.34 \pm 0.21 to 2.52 \pm 0.21) and an increase of ¹⁴CO₂ produced from [2-¹⁴C]pyruvate (from 0.75 \pm 0.05 to 1.10 \pm 0.05) by soleus muscle incubated for 1 h. The values are expressed as μ mol/h per mg protein and presented as mean \pm S.E.M. of six determinations from two experiments.

3.2. Effect of palmitate on insulin-induced protein phosphorylation

As one should expect, an increase in tyrosine phosphorylation of a band corresponding to pp185 was obtained by im-

muno blotting of the whole extract after insulin stimulation. pp185 is known to contain at least two well characterized proteins, IRS-1 and IRS-2. IRS-1 is the major component of this band [41,42].

IRS-1 protein level remained unchanged (Fig. 2A) but 100 μ M palmitate induced a decrease in insulin-stimulated IRS-1/2 tyrosine phosphorylation already after 5 min incubation (Fig. 2B). In the absence of insulin, palmitate had no effect on this protein phosphorylation after 5 min incubation (data not shown). Since stimulation with insulin results in activation of the PI3-K/Akt [43–45] and MAP kinase pathways [46], immunoblotting with anti-phosphoserine(473)-Akt and anti-phospho-p44/42 MAP kinase antibodies was then performed. Insulin-induced Akt serine phosphorylation was reduced in isolated muscles incubated with palmitate. The decrease was 39% ($P < 0.01$) after 5 min and reached 51% ($P < 0.001$) after 60 min (Fig. 2C). The insulin-induced phosphorylation of p44/42 MAP kinases was also reduced by palmitate. The reduction was 64% after 30 min ($P < 0.05$) and reached 88% after 60 min ($P < 0.001$) of incubation with palmitate (Fig. 2D).

3.3. Evidence that palmitate metabolism is required to promote its effect on glycogen synthesis

Bromopalmitate (a non-metabolizable analogue) in contrast to palmitate reduced insulin-stimulated glycogen synthesis by 27% ($P < 0.001$) (Fig. 3). The glucose 6-phosphate intracellu-

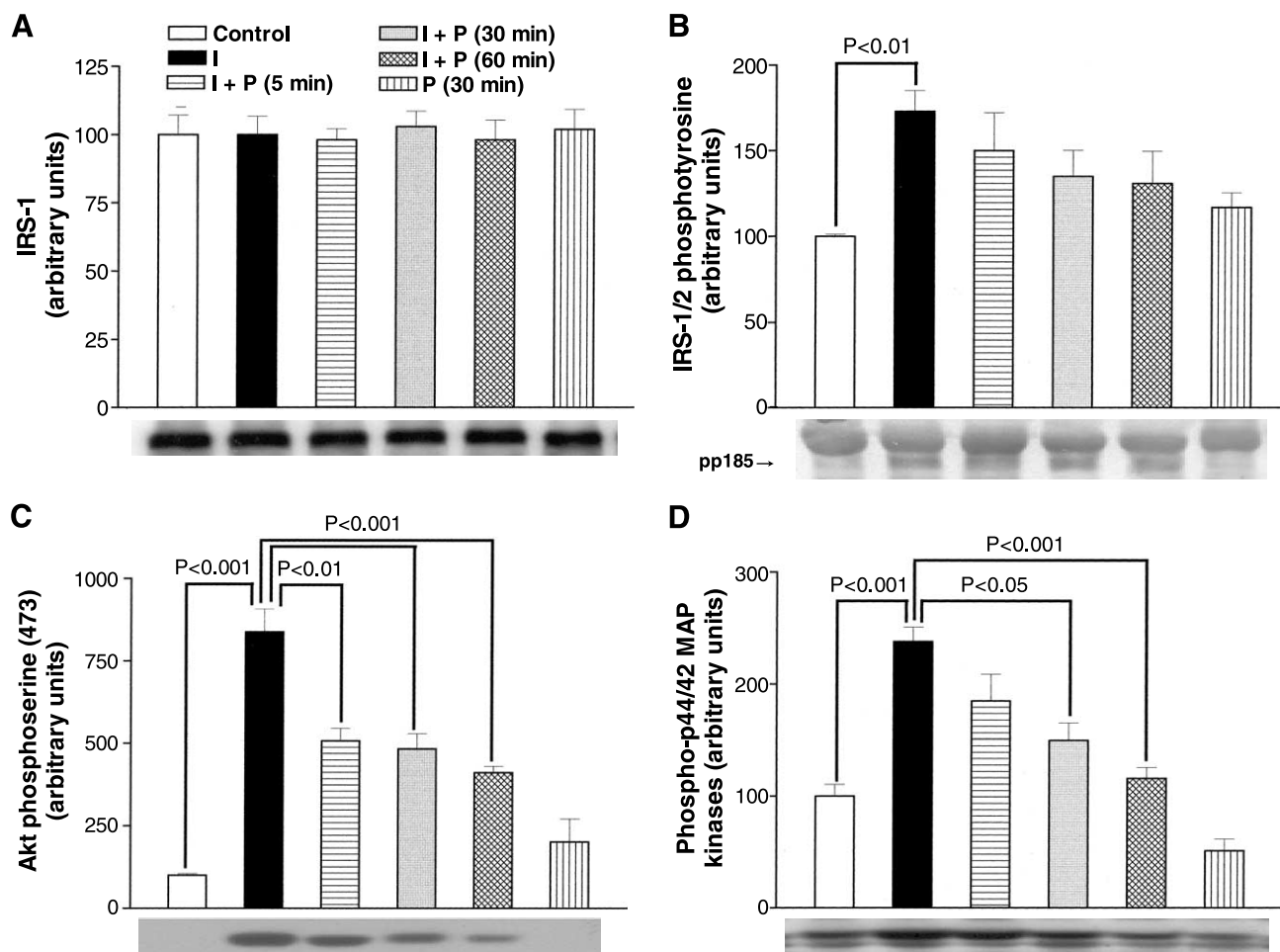


Fig. 2. Effect of palmitate (P) on total IRS-1 (A) and on insulin-stimulated phosphorylation of IRS-1/2 (B), Akt (C) and p44/42 MAP kinases (D) in soleus muscle. Muscles were incubated for 5, 30 or 60 min in the presence of 100 μ M palmitate. Insulin (I) was added to the medium at 10 mU/ml and the muscles incubated for 5 min more. Some muscles were incubated for 5 min in the absence (control group) or presence of 10 mU/ml insulin (insulin group) and others for 30 min in the presence of 100 μ M palmitate. At the end of the incubation, muscles were homogenized in extraction buffer at 100°C and centrifuged as described in Section 2. Aliquots of proteins of the muscle lysates (75 μ g) were resolved in 6% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. IRS-1, IRS-1/2 phosphotyrosine, Akt phosphoserine(473) and phospho-p44/42 MAP kinases were visualized by immunoblotting using specific IRS-1, phosphotyrosine, phosphoserine(473)-Akt and phospho-p44/42 MAP kinase antibodies, respectively. Data are presented as mean \pm S.E.M. of three experiments ($n = 6$).

lar content was not altered by palmitate itself (Fig. 4). However, the simultaneous incubation of 100 μ M palmitate with 10 mU/ml insulin induced a significant increase (by 27%) of the content of this metabolite as compared with the insulin group ($P < 0.01$) (Fig. 4).

To determine if oxidation of palmitate was correlated with insulin-stimulated glycogen synthesis, both measurements were carried out concomitantly in soleus muscle incubated for 15, 30 and 60 min in the presence of 10 mU/ml insulin and 100 μ M palmitate. [14 C]Glycogen synthesis and [U- 14 C]palmitate decarboxylation were highly correlated: $r^2 = 0.99$.

4. Discussion

Palmitate increased insulin-stimulated glycogen synthesis and decreased insulin-stimulated glycolytic flux (lactate production). Incubation in the presence of insulin and palmitate raised the glucose 6-phosphate content in the muscles (Fig. 4) as postulated by the Randle cycle [4]. These results, in association with the fact that insulin-stimulated 2-deoxy-D-glucose

uptake was not altered by the fatty acid, suggest that palmitate leads to glucose accumulation as glycogen rather than being utilized through glycolysis. Palmitate did not inhibit glucose decarboxylation and instead increased this parameter bluntly (Fig. 1C). Pyruvate generated from glucose is converted into CO_2 through pyruvate dehydrogenase and the Krebs cycle entering via the pyruvate carboxylase reaction [31,47]. In order to estimate the contribution of both decarboxylation sites (pyruvate dehydrogenase and Krebs cycle), measurements of $^{14}\text{CO}_2$ production from [^{14}C]- and [^{14}C]pyruvate were carried out. [^{14}C]Pyruvate is decarboxylated through the tricarboxylic acid cycle only in opposition to [^{14}C]pyruvate, which produces $^{14}\text{CO}_2$ in both sites. In the presence of insulin, palmitate decreased the production of $^{14}\text{CO}_2$ from [^{14}C]pyruvate and enhanced $^{14}\text{CO}_2$ formation from [^{14}C]pyruvate. An increase in acetyl-CoA content from palmitate oxidation leads to inhibition of pyruvate dehydrogenase activity, decreasing the conversion of pyruvate to acetyl-CoA, but also enhances pyruvate carboxylase activity, raising the conversion of pyruvate to oxaloacetate that enters the Krebs cycle being then oxidized.

The differences between our studies and those of others [48–50] may be due to the concentrations of palmitate used. While the studies that found reduction in insulin actions utilized supraphysiological concentrations of fatty acids, from 1 to 2.8 mM [48–50], in the present study plasma physiological concentrations of palmitate were used: 50 and 100 μ M.

The effects of palmitate on some of the proteins involved in the mechanism of action of the hormone were studied. Palmitate reduced insulin-stimulated phosphorylation of IRS-1/2, Akt, and p44/42 MAP kinases. These results suggest that the increased insulin-stimulated glycogen synthesis by palmitate is not related to activation of these proteins. Results of the total IRS-1 suggest that the lower phosphorylation of this protein induced by the fatty acid was not due to inhibition of protein synthesis. Whether lower insulin-stimulated IRS-1 tyrosine phosphorylation in the presence of palmitate is due to inhibition of insulin receptor tyrosine kinase activity, increased serine/threonine phosphorylation of IRS-1 or stimulation of phosphotyrosine phosphatase activity remains to be investigated. These three hypotheses have been associated with decreased insulin-induced tyrosine phosphorylation in studies using cultured cells and animal tissues [51–54].

Simoneau et al. [55] observed that increased plasma FFA levels in humans subjected to euglycemic-hyperinsulinemic glucose clamp decrease FFA oxidation and increase FFA storage in skeletal muscle. However, other studies show increased expression of fatty acid metabolism genes in muscle due to infusion of fatty acids [56]. Bromopalmitate is a competitive inhibitor of carnitine palmitoyltransferase-1 [31,57,58] and so of FFA oxidation. In opposition to palmitate, bromopalmitate reduced insulin-stimulated glycogen synthesis. This observation associated with the high correlation between increased insulin-stimulated glycogen synthesis and decarboxylation of palmitate and the accumulation of glucose 6-phosphate induced by palmitate support the proposition that palmitate metabolism is required to potentiate insulin-stimulated glycogen synthesis.

In summary, palmitate acutely increases insulin-stimulated glycogen synthesis and decreases insulin-stimulated glycolytic

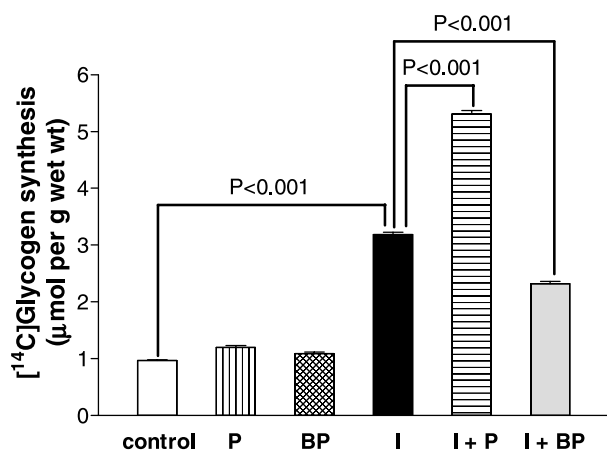


Fig. 3. Effect of bromopalmitate (BP) on [14 C]glycogen synthesis in soleus muscle. Muscles were incubated in Krebs–Ringer bicarbonate buffer, containing 5.6 mM glucose and 0.3 μ Ci/ml D-[14 C]glucose, for 1 h in the absence or presence of 10 mU/ml insulin (I) and/or 100 μ M palmitate (P) or BP. [14 C]Glycogen synthesis was determined as described by Leighton and Cooper [28]. Data are presented as mean \pm S.E.M. ($n = 15$).

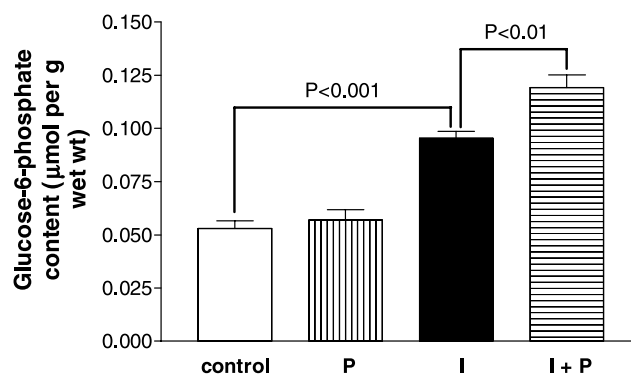


Fig. 4. Effect of palmitate (P) on glucose 6-phosphate intracellular content in incubated soleus muscle. To determine the effect of P, muscles were incubated in Krebs–Ringer bicarbonate buffer, containing 5.6 mM glucose, for 1 h in the absence or presence of 10 mU/ml insulin (I) and/or 100 μ M P. Muscles were then frozen in liquid N_2 and the glucose 6-phosphate content was determined as described by Lang and Michal [40]. Data are presented as mean \pm S.E.M. ($n = 12$).

flux in skeletal muscle by a mechanism that requires its metabolism. These effects may occur through the Randle cycle. Reduction in the insulin-stimulated phosphorylation of IRS-1/2, Akt and p44/42 MAP kinases by palmitate does not decrease insulin-stimulated glycogen synthesis acutely but it may be involved in the onset of insulin resistance in conditions of chronic exposure to high plasma FFA levels.

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