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Research paper

Signaling pathways of kaempferol-3-neohesperidoside in glycogen synthesis in rat soleus muscle

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ABSTRACT

Kaempferol 3-neohesperidoside is one of the several compounds that have been reported to have insulin-like properties in terms of glucose lowering. We studied the effect of kaempferol 3-neohesperidoside in glycogen synthesis in rat soleus muscle through the incorporation of ^{14}C-D-glucose in glycogen. Kaempferol 3-neohesperidoside stimulates glycogen synthesis in rat soleus muscle by approximately 2.38-fold. Insulin at 100 nM showed a stimulatory effect on glycogen synthesis when compared with the control group. The stimulatory effect of kaempferol 3-neophesperidoside on glycogen synthesis was inhibited by wortmannin, the phosphatidylinositol 3-kinase (PI3K) inhibitor, and enhanced by lithium chloride, a glycogen synthase kinase 3 (GSK-3) inhibitor. Moreover, the stimulatory effect of kaempferol 3-neohesperidoside was also nullified by PD98059, a specific inhibitor of mitogen-activated protein kinase (MEK) and by calyculin A, an inhibitor of protein phosphatase 1 (PP1) activity. It was concluded that the PI3K – GSK-3 pathway and MAPK – PP1 pathway are involved in the stimulatory kaempferol 3-neohesperidoside effect on glycogen synthesis in rat soleus muscle.

1. Introduction

Insulin is the most important hormone that regulates energy metabolism. It mediates a wide spectrum of biological responses including synthesis and storage of carbohydrates, lipids and proteins as well as the inhibition of catabolism [1,2]. In mammalian tissues, carbohydrate is stored mainly in the form of glycogen and the major sites of glycogen deposition are skeletal muscle and the liver [3]. An absolute or relative lack of insulin, as in the case of diabetes, leads to severe dysfunction and deregulation of insulin signaling in target tissues such as the liver, adipose tissue and muscles. Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both [2]. In recent years, there has been a growing interest in hypoglycemic agents from natural products, especially those derived from plants [4–10]. Substances that mimic insulin action are of interest since they can act efficiently in the alleviation of insulin resistance and diabetes [10,11].

The flavonoids are a group of low-molecular-weight polyphenolic substances, qualitatively and quantitatively one of the largest groups of natural products known. Besides their roles in plants, flavonoids are important components in the human diet and are found in fruits, vegetables, seeds, nuts, grains, spices and beverages [12,13]. They have a broad range of biological activities and numerous studies have been carried out on their potential role in the treatment and prevention of diseases and especially of diabetes [13–15]. Many studies have demonstrated the hypoglycemic effects of flavonoids [16–18] as well as their action in glucose uptake [16–19], glycogen metabolism [20–22] and gluconeogenic enzyme activities [23–25].

We have previously demonstrated the acute hypoglycemic effect of isolated flavonoids as well as flavonoid-enriched fractions [5,6,26]. Recently, it was reported that kampferitrin, the major flavonoid found in *Bauhinia forficata* leaves, was able to reduce glycemia and stimulate glucose uptake in rat soleus muscle as efficiently as insulin [19]. In addition, studies with kaempferol 3-neohesperidoside, alone or complexed with vanadium IV, isolated from *Cyathea phalerata* Mart. (Cyatheaceae) stems and structurally similar to kaempferitrin, showed a significant hypoglycemic effect in diabetic rats [6]. This

Abbreviations: PI3K, phosphatidylinositol 3-kinase; GSK-3, glycogen synthase kinase 3; MEK/MAPKK, mitogen-activated protein kinase kinase; PP1, protein phosphatase 1; PKC, protein kinase C; GLUT4, glucose transporter 4; GS, glycogen synthase; LiCl, lithium chloride; MAPK, mitogen-activated protein kinase; p90^{rsk2}, p90 ribossomal S6 kinase; PKB, protein kinase B; PDK, protein kinase dependent on 3-phosphoinositides.

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flavonoid was able to increase glucose uptake as well as glycogen content in rat soleus muscle. The mechanism through which kaempferol 3-neohesperidoside stimulates glucose uptake is probably mediated by phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) pathways and, at least in part, is independent of mitogen-activated protein kinase (MEK) pathways and the synthesis of new glucose transporters [27].

Glycogen synthesis in skeletal muscle has a key role in the control of blood glucose levels by insulin since most of the postprandial glucose uptake occurs in this tissue. Most of the glucose that enters muscle fibers through glucose transporter 4 (GLUT4) in response to insulin is converted into glycogen. This hormonal effect involves activation of glycogen synthase (GS), the enzyme that catalyzes the rate-limiting step in the conversion of intracellular glucose to glycogen [3,28]. Insulin activates glycogen synthase by promoting dephosphorylation of several sites of the enzyme through the inhibition or stimulation of protein kinases and phosphatases, respectively [3,29]. Moreover, insulin also regulates glycogen synthase activation by controlling the uptake and transport by GLUT4 of glucose and by regulating the phosphorylation and activation states of enzymes involved in the synthesis and degradation of glycogen [3,28]. Based on this, the aim of this study was to investigate the effect of kaempferol 3-neohesperidoside on glycogen synthesis in rat soleus muscle and the signaling pathways involved in its mechanisms of action.

2. Material and methods

2.1. Materials

Regular human insulin (Biohulin) was obtained from Biobrás, Bioquímica do Brazil S/A (Águas Claras, MG, Brazil). Wortmannin, inhibitor of phosphatidylinositol 3-kinase (PI3K); calyculin A, inhibitor of protein phosphatase 1 (PP1); PD98059, inhibitor of mitogen-activated protein kinase (MEK) and lithium chloride, inhibitor of glycogen synthase kinase 3 (GSK-3) were purchased from Sigma–Aldrich Co. D – [^{14}C (U)] – glucose (^{14}C-glucose), specific activity 9.25 GBq/mmol and biodegradable liquid scintillation cocktails were obtained from Perkin–Elmer Life and Analytical Sciences (Boston, MA, USA). Salts and solvents were purchased from Merck AG (Darmstadt, Germany).

2.2. Plant material

C. phalerata Mart. (Cyatheaceae) was collected in March 2002 in Palhoça, Brazil, and identified by Prof. Lana da Silva Sylvestre. A voucher specimen (RBR 4287) has been deposited in the herbarium of the Botany Department at the Universidade Federal Rural do Rio de Janeiro, Seropedica, Brazil. The process of extraction and isolation of kaempferol 3-neohesperidoside was carried out as described in [27]. The flavonoid used in this study was dissolved in 1% EtOH/H_2O solution and stored aliquotted at – 20 degree Celsius.

2.3. Experimental animals

Male Wistar rats weighing 190–210 g from the Central Animal House-UFSC were used. The rats were housed in plastic cages in an air-conditioned animal room and fed on pellets with free access to tap water. Room temperature was controlled at 21 degree Celsius with a 12 h light:12 h dark cycle. Animals described as fasted had been deprived of food for 16 h but allowed free access to water. All the animals were monitored and maintained in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP007).

2.4. Glycogen synthesis in rat soleus muscle

The assays of ^{14}C-glucose incorporation into glycogen were conducted as described in [19,30] with modifications. Slices (strips) of soleus muscle from normal rats were distributed (alternately left and right) between control and treated groups. Muscles were dissected, weighed, preincubated and incubated at 37 degree Celsius in Krebs Ringer-bicarbonate (KRb) buffer comprising 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ plus 1% BSA and 5 mM D-glucose and bubbled with O_2/CO_2 (95%:5%, v/v) until pH 7.4 was reached. Kaempferol 3-neohesperidoside (0.1; 1 and 10 μM) and insulin (10 and 100 nM) were added to the preincubation (30 min) and incubation (60 min) media in the presence or absence of 100 nM wortmannin, 5 nM calyculin A, 50 μM PD98059 or 50 mM lithium chloride. ^{14}C-glucose (0.15 μCi/ml) was added to each sample during the incubation period. After incubation, the muscle samples were removed, washed in cold KRb and dried on filter paper. The muscle samples were homogenized in 0.5 N KOH, and boiled at 100 degree Celsius for 20 min, with occasional stirring. After cooling, 95% ethanol was added to the samples, which were heated to boiling followed by cooling in an ice bath for 20 min to allow glycogen precipitation. The homogenates were centrifuged at 664 g for 15 min, the supernatant was discarded and pellets resolubilized in water. Aliquots (30 μl) of the samples were placed in liquid scintillation vials in an LKB rack, on a beta liquid scintillation spectrometer (model 1215; EG and G-Wallac, Turku, Finland), for the radioactivity measurements. The results were expressed as pmol glycosyl units incorporated in glycogen. mg tissue^{-1} h^{-1}.

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. One or two-way analysis of variance (ANOVA) followed by the Bonferroni post-test was used to identify significantly different groups. Differences were considered to be significant at $P \leq 0.05$.

3. Results

3.1. Studies on kaempferol 3-neohesperidoside and insulin in glycogen synthesis

As shown in Fig. 1 kaempferol 3-neohesperidoside (Fig. 1A) caused a significant increase in the *in vitro* glycogen synthesis in soleus muscle compared with the control (Fig. 1B). In percentage terms, the effect of the flavonoid at 1 μM was around 140% compared with the control. The insulin concentrations used were those previously reported by Jorge et al. (2004). As expected, insulin efficiently stimulated glucose incorporation into glycogen. This effect of insulin (10 and 100 nM) increased around 2-fold when compared with the control group (Fig. 2). The effect of kaempferol 3-neohesperidoside represents approximately twice that of insulin stimulation (10 nM) in glycogen synthesis, although the effective dose of the compound used was higher than that used for insulin.

3.2. Effect of various inhibitors on the stimulatory action of kaempferol 3-neohesperidoside in glycogen synthesis in rat soleus muscle

To determine the mechanism by which kaempferol 3-neohesperidoside induced glycogen synthesis in the soleus muscle, we performed the glycogen synthesis assay with calyculin A, a specific inhibitor of PP1 activity, PD98059, a specific inhibitor of MEK, wortmannin, a specific inhibitor of PI3K, or LiCl, a known inhibitor of GSK-3. The inhibitor concentrations used were those previously