Insulinomimetic Effect of Kaempferol 3-Neohesperidoside on the Rat Soleus Muscle

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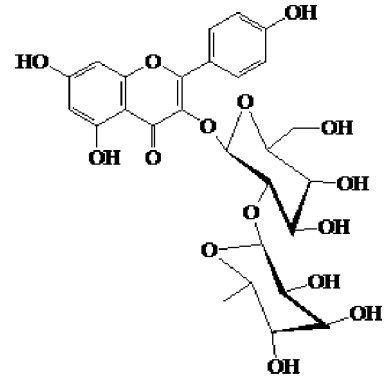
A stimulatory effect of kaempferol 3-neohesperidoside (**1**) on glucose uptake (35% and 21%) was observed when the rat soleus muscle was incubated with 1 and 100 nM of this flavonoid glycoside, respectively. The concentration–response curve of insulin showed a stimulatory effect at 3.5 and 7.0 nM (42% and 50%) on glucose uptake when compared with the control group. The effect of **1** on glucose uptake was completely nullified by pretreatment with LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K), and RO318220, an inhibitor of protein kinase C (PKC). However, no significant change occurred on glucose uptake stimulated by **1** when muscles were pretreated with PD98059, an inhibitor of mitogen-activated protein kinase (MEK), and cycloheximide, an inhibitor of protein synthesis. Compound **1** and insulin (7 nM) did not show a synergistic effect on glucose uptake. Additionally, 100 mg/kg of **1** by oral gavage was able to increase glycogen content in the muscle. These results suggest that **1** stimulates glucose uptake in the rat soleus muscle via the PI3K and PKC pathways and, at least in part, independently of MEK pathways and the synthesis of new glucose transporters.

Diabetes mellitus is a disease characterized by chronic hyperglycemia that can lead to several complications related to cardiovascular disease, renal failure, blindness, and neurological disorders.[1] In recent years, there has been a growing interest in hypoglycemic agents from natural products, especially those derived from plants. They represent an alternative method for diabetes therapy because most of the hypoglycemic agents and insulin have some side effects and fail to significantly alter the course of diabetic complications.[2,3]

Flavonoids are phenolic compounds found widely in fruits and vegetables.[4] The therapeutic applications of flavonoids in the treatment and prevention of diseases in humans have been demonstrated in several recent studies. Among the best documented therapeutic uses of the flavonoids include the treatment and prevention of allergy, asthma, and inflammation.[5] Beneficial effects of flavonoids in diseases such as cancer,[6,7] cardiovascular diseases,[7,8] diabetic cataracts,[9] and cirrhosis[10] have also been reported. Several flavonoids exert an effect on glucose transport and on insulin-receptor function in vitro and could affect diabetes.[4,11–14] We have previously reported the acute hypoglycemic effect of isolated flavonoids as well as in flavonoid-enriched fractions in diabetic rats.[15–17] Recently, it was demonstrated that kaempferitrin, the major flavonoid found in *Bauhinia forficata* leaves, is able to diminish serum glucose level and increase glucose uptake in the rat soleus muscle as efficiently as insulin.[13] Also, kaempferol 3-neohesperidoside (**1**) isolated from *Cyathea phalerata* Mart. (Cyatheaceae) stems showed a significant hypoglycemic effect in diabetic rats.[16]

Insulin mediates a wide spectrum of biological responses including stimulation of glucose uptake, glycogen, lipid, and protein synthesis, antilipolysis, activation of transcription of specific genes, and modulation of cellular growth and differentiation.[19] Insulin stimulates glucose uptake via multiple signaling pathways. The binding of insulin to its receptor activates the intrinsic receptor tyrosine kinase, which results in autophosphorylation and recruitment of substrates, such as insulin receptor substrate (IRS) proteins and Cbl protein.[1] Specific tyrosine residues on the IRS proteins



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serve as docking sites for proteins that contain SH2 domains, such as the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). PI3K catalyzes the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), an allosteric activator of phosphoinositide-dependent kinase (PDK). Targets of PDK include protein kinase B (PKB) and the atypical protein kinase C (aPKC) isoforms, which, when activated via phosphorylation, stimulate the translocation of GLUT4-containing vesicles to the plasma membrane.[20] On the basis of a prompt and efficient hypoglycemic effect of kaempferol 3-neohesperidoside (**1**) compared with exogenous insulin in diabetic rats,[16] the aim of the present work was to study the acute effects of **1** on ^{14}C-glucose uptake and the mechanism of action of this flavonoid in a target tissue of insulin, the soleus muscle.

Results and Discussion

Studies of Kaempferol 3-Neohesperidoside (1) and Insulin Effects on ^{14}C-Glucose Uptake in the Rat Soleus Muscle. Figure 1 shows the in vitro effect of kaempferol 3-neohesperidoside (**1**) (0.0001, 0.001, 0.1, 1, 10, 20, 200, 520, 1040, 1580, 2080 μM) on glucose uptake in the rat soleus muscle following a 60 min incubation. Concentrations of **1** ranging from 0.0001 to 2080 μM on the stimulatory effect on glucose uptake showed a bell-shaped response curve typical of that of the hormonal response.[13,23,24] The stimulatory effect of **1** was significant at 1 and 100 nM and represented 35% and 21% of glucose uptake compared to the basal

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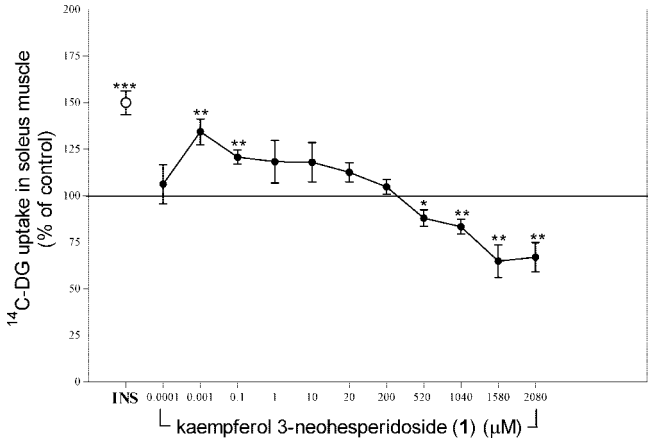

Figure 1. Concentration–response curve of kaempferol 3-neohesperidoside (**1**) on ^{14}C-glucose uptake in the rat soleus muscle. Insulin (INS) = 7 nM. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± SEM; $n = 6$ in duplicate for each group. ***Significant at $p \leq 0.001$, **$p \leq 0.01$, and *$p \leq 0.05$ in relation to control group (100% of ^{14}C-glucose uptake).

Table 1. Insulin Concentration–Response Curve on ^{14}C-Glucose Uptake in Rat Soleus Muscle from Normal Rats[a]

insulin (nM)	^{14}C-glucose uptake (tissue/medium ratio)
0.0	1.02 ± 0.03
0.007	0.83 ± 0.02
0.07	0.94 ± 0.02
0.7	1.09 ± 0.07
3.5	1.45 ± 0.09[b]
7	1.53 ± 0.1[b,c]
35	1.120 ± 0.08
70	0.88 ± 0.04

[a] Preincubation = 30 min; incubation time = 60 min. Values are expressed as mean ± SEM; $n = 6$ in duplicate for each group. [b] Significant at $p \leq 0.001$ in relation to control group. [c] Significant at $p \leq 0.01$ between concentrations.

value at 60 min. Table 1 shows the concentration–response effect of insulin on [U−^{14}C]-2-deoxy-D-glucose (^{14}C-DG uptake) in the soleus muscle after 60 min of incubation. With this approach, the best stimulatory effect of insulin (7 nM) on glucose uptake was around 50%. In percentage terms, the compound (1 nM) was as effective as insulin (7 nM). We have previously demonstrated the efficient effect of **1** on serum glucose lowering when compared with insulin and with a similar flavonoid, kaempferitrin.[16] Taken together, these results clearly reinforce the insulinomimetic potential role of **1** on glucose uptake in one of the insulin target tissues, the rat soleus muscle.

Effect of Various Inhibitors on the Stimulatory Action of Kaempferol 3-Neohesperidoside (1) on ^{14}C-Glucose Uptake in the Rat Soleus Muscle. To determine the mechanism by which kaempferol 3-neohesperidoside (**1**) induced glucose uptake in the soleus muscle, we performed the glucose uptake assay with 10 μM LY294002, a specific inhibitor of PI3K,[26–28] 40 μM RO318220, a specific inhibitor of PKC,[27,29,30] 50 μM PD98059, an inhibitor of MEK,[27,31] or 0.35 mM cycloheximide, an inhibitor of protein synthesis.[25] The LY294002, RO318220, PD98059, and cycloheximide concentrations used were those that have previously resulted in inhibition in cell and tissue assays.[25–30,32] Figure 2 shows that the promotion of glucose uptake by 1 nM **1** was completely inhibited by LY294002 and RO318220 pretreatment, whereas no change was observed with PD98059 and cycloheximide pretreatment. When only LY294002, RO318220, PD98059, or cycloheximide was added to the muscle samples, no significant change resulted compared with the glucose uptake in the control group. Thus, the stimulatory effect of **1** on glucose uptake is mediated, at

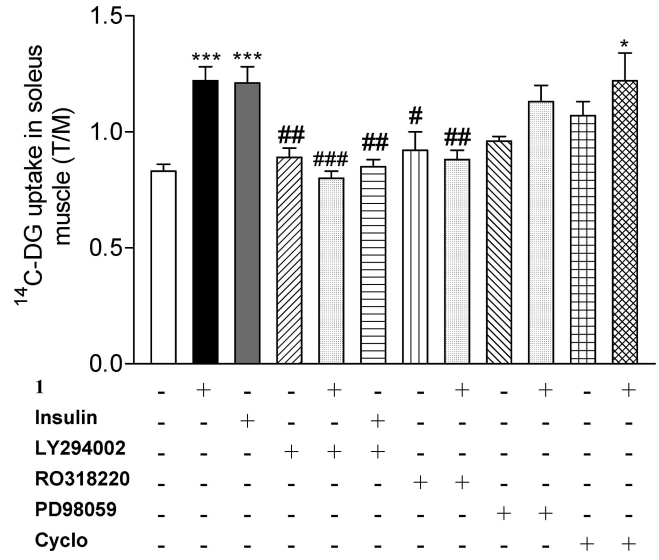

Figure 2. Effect of enzyme inhibitors on the stimulatory action of kaempferol 3-neohesperidoside (**1**) on ^{14}C-glucose uptake in the rat soleus muscle with 10 μM LY294002, 50 μM RO318220, 40 μM PD98059, 0.35 mM cycloheximide, 1 nM **1**, and 7 nM insulin used. Control group = no treatment. Signal (+) and (−) indicates presence and absence, respectively, of each substance in the incubation medium. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± SEM; $n = 6$ in duplicate for each group. ***Significant at $p \leq 0.001$ and *$p \leq 0.05$ in relation to control group. ###Significant at $p \leq 0.001$, ##$p \leq 0.01$, and #$p \leq 0.05$ in relation to kaempferol 3-neohesperidoside group.

least in part, through insulin signal transduction involving the PI3K and PKC pathways and is independent of MEK and the synthesis of new transporters (GLUT). Taking these results together, we can suggest an insulinomimetic effect of this flavonoid since it was effective as a hypoglycemic agent[16] and increased glucose uptake in a short-term exposure in an insulin target tissue. The present results are in accord with the literature concerning the key role of PI3K and GLUT4 translocation regulated by insulin.[20,33,34] Moreover, it was also demonstrated for platelet-derived growth factor, epidermal growth factor,[35,36] and leucine.[37] Additionally, the stimulatory effect of kaempferol 3-neohesperidoside (**1**) on glucose uptake seems to activate the GLUTs translocation to the membrane through atypical PKC isoforms, as has been demonstrated with insulin, leucine, and puerarin.[26,38,39] The present results reinforce the involvement of atypical PKC isoforms since RO318220 (40 μM) is able to block all PKC isoforms.[40]

Comparative Effect of Kaempferol 3-Neohesperidoside (1) and Insulin on ^{14}C-Glucose Uptake in the Rat Soleus Muscle. Figure 3 shows the stimulation glucose uptake by kaempferol 3-neohesperidoside (**1**) (1 nM) compared to insulin (7 nM) in the rat soleus muscle. In percentage terms, the compound was as effective as insulin, and when the effects at these concentrations were compared, **1** was more potent than insulin. The combination of the highest stimulatory concentration of **1** and insulin did not cause an additive effect on glucose uptake in the rat soleus muscle. These results clearly demonstrate that the effect of **1** on glucose uptake is partly through the same insulin signal transducing pathway. To our knowledge, this is the first report of kaempferol 3-neohesperidoside (**1**) on glucose uptake stimulatory effect in a target tissue of insulin. There are various molecular mechanisms by which this stimulation of glucose uptake could take place. Pinent et al. showed that the action of grape seed-derived procyanidins on glucose uptake in insulin-sensitive cell lines involves insulin mechanisms, since simultaneous treatment with saturating doses

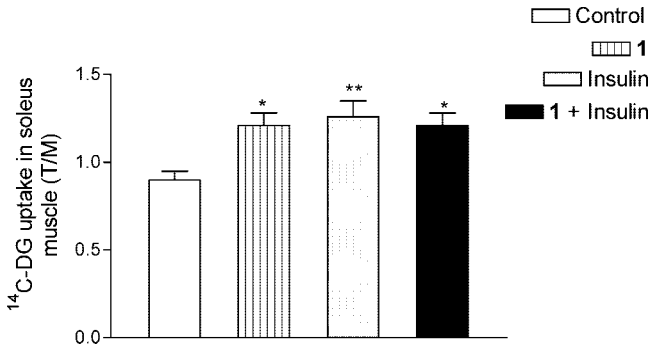

Figure 3. Comparative effect of kaempferol 3-neohesperidoside (**1**) (1 nM) and insulin (7 nM) on ^{14}C-glucose uptake in the rat soleus muscle. Preincubation time = 30 min; incubation time = 60 min. Values are expressed as mean ± SEM; $n = 6$ in duplicate for each group. **Significant at $p \leq 0.01$ and *$p \leq 0.05$ in relation to control group.

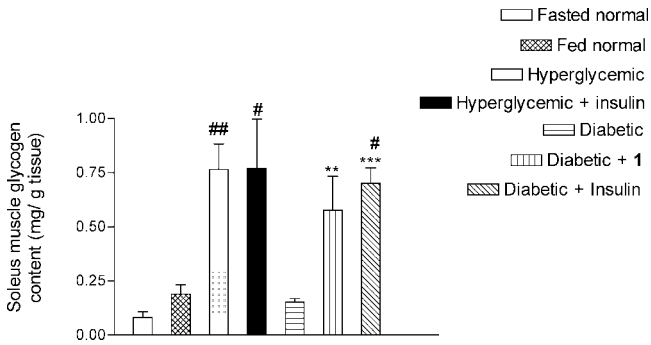

Figure 4. Effect of kaempferol 3-neohesperidoside (**1**) on muscle glycogen content in fasted diabetic rats. Insulin 3.5 nM by ip route; **1** 100 mg/kg administered by oral gavage. ##Significant at $p \leq 0.01$ and #$p \leq 0.05$ in relation to normal group. ***Significant at $p \leq 0.001$ and **$p \leq 0.01$ in relation to diabetic group. Values are expressed as mean ± SEM; $n = 6$ in triplicate for each group.

of insulin and procyanidins did not cause an additive effect on glucose uptake.[4]

Effect of Kaempferol 3-Neohesperidoside (1) on Glycogen Content in the Soleus Muscle from Diabetic Rats. Figure 4 shows that the soleus muscle glycogen content was increased significantly 24 h after the administration of **1** (100 mg/kg) in diabetic rats compared with fed, fasted normal, and diabetic control rats. Additionally, the known stimulatory effect of insulin on glycogen storage was observed after 24 h (461%) of insulin treatment in diabetic rats compared with the untreated diabetic rats. Moreover, the glycogen content in hyperglycemic rats (4 g/kg glucose) treated with insulin (3.5 nM) was also increased when compared with fed and fasted normal rats. In percentage terms, the effect of **1** on glycogen content of diabetic rats represents an increase of 380% compared with the untreated diabetic group. On the other hand, liver glycogen content was not modified after 24 h of treatment with **1**, contrasting with fed normal and hyperglycemic control groups (data not shown).

In addition to the pharmacological effects mentioned previously,[5–10] flavonoid *O*- and *C*-glycosides have been associated with carbohydrate metabolism.[41] Recent studies have indicated that **1** is an effective hypoglycemic agent through the oral as well as the intraperitoneal route in alloxan-induced diabetic rats.[16] This observation warrants further investigations of the possible effects of **1** on glucose uptake to elucidate its hypoglycemic activity. As hypothesized in Figure S1 (Supporting Information), the prompt efficiency of **1** on the stimulatory effect on ^{14}C-DG uptake in muscle as well as its hypoglycemic effect constitutes the first evidence indicating that the acute effect of this compound on lowering blood glucose may occur as a consequence of the altered intrinsic activity of the glucose transporter (transporters translocation) not involving directly the synthesis of new carriers.

Experimental Section

General Experimental Procedures. Alloxan was purchased from Sigma Chemical Company (St. Louis, MO). Regular human insulin (Biohulin) was obtained from Biobrás Bioquímica do Brasil S/A (Águas Claras, MG, Brazil). Inhibitors of PI3K, LY294002, PKC, RO318220, mitogen-activated protein kinase kinase (MEK), PD98059, and protein synthesis, cycloheximide, were purchased from Sigma-Aldrich Co. [U−^{14}C]-2-Deoxy-D-glucose (^{14}C-DG), specific activity 7.4 GBq/mmol, and biodegradable liquid scintillation were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA). Salts and solvents were purchased from Merck AG (Darmstadt, Germany).

Plant Material. *Cyathea phalerata* Mart. (Cyatheaceae) was collected in March 2002 at 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.

Extraction and Isolation. Fresh *Cyathea phalerata* stems (9.18 kg) were cut into small pieces and extracted with 8:2 ethanol–water by maceration at room temperature. The hydroalcoholic extract was concentrated to the desired level, and an aqueous suspension was defatted with hexane and extracted with EtOAc. The EtOAc fraction was subjected to column chromatography over silica gel 60, eluting with a gradient of hexane−EtOAc−EtOH. Fractions containing kaempferol 3-neohesperidoside (**1**) were combined and further purified by flash chromatography on silica gel to give the pure compound, which was identified by spectroscopic analysis (^{1}H NMR, ^{13}C NMR, HMBC, and HMQC) and compared with literature data.[21,22] The interglycosidic linkage and the sequential arrangement of the sugars were deduced from the NMR spectroscopic data, including COSY, HMQC, and HMBC.

Kaempferol 3-neohesperidoside (1): yellow crystals from acetone; mp 183.4–185.0 °C; ^{1}H NMR (400 MHz, C_5D_5N) aglycone δ 6.25 (1H, d, $J = 2.0$ Hz, H-6), 6.47 (1H, $J = 2.0$ Hz, H-8), 7.03 (2H, d, J 26> 8.3 Hz, H-3′/H-5′), 7.87 (2H, d, $J = 8.3$ Hz, H-2′/H-6′), 12.64 (1H, s, OH-5); sugar moiety δ 1.52 (3H, d, $J = 6.1$ Hz, Me-6‴), 3.77 (1H, brt, H-5‴), 4.09 (1H, brt, $J = 7.8$ Hz, H-2‴), 4.22 (1H, m, H-3‴), 4.30 (1H, m, H-4‴), 4.35–4.41 (2H, m, H-5‴/H-6‴), 4.46 (1H, brt, $J = 9.2$ Hz, H-4‴), 4.79 (1H, dd, $J = 9.2$ and 3.0 Hz, H-3‴), 5.24 (1H, d, $J = 7.8$, H-1″), 6.12 (1H, d, $J = 1.3$ Hz, H-1‴); ^{13}C NMR (100 MHz, C_5D_5N) δ 178.7 (C-4), 165.8 (C-7), 162.7 (C-5), 161.5 (C-4′), 157.5 (C-7), 147.0 (C-2), 135.8 (C-3), 131.2 (C-2′/C-6′), 121.4 (C-1′), 116.1 (C-3′/C-5′), 106.4 (C-1‴), 105.2 (C-10), 103.7 (C-1‴), 99.6 (C-6), 94.4 (C-8), 84.2 (C-4‴), 78.4 (C-3″), 78.3 (C-5″), 76.1 (C-2″), 72.2 (C-3‴), 71.4 (C-2‴), 71.1 (C-4″), 70.1 (C-5‴) 62.3 (C-6″), 18.0 (C-6‴), *anal.* C 54.52%, H.5.04%, calcd for $C_{27}H_{30}O_{15}$, C 54.545%, H 5.050%.

Experimental Animals. Male Wistar rats weighing 170–190 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 °C 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.

Diabetic Animals. Rats were made diabetic by a single intravenous injection of alloxan monohydrate 5% (w/v) in a saline solution at a dose of 50 mg/kg body weight. Blood samples were collected 3 days later, and glucose levels were determined to confirm the development of type 1 diabetes.[13,15–18]

Kaempferol 3-Neohesperidoside (1) and Insulin Treatments. Normal, hyperglycemic, and diabetic rats were divided into control and treated groups. Rats in the control groups were randomly divided into groups of six rats: normal fed rats; normal fasted rats (overnight); normal hyperglycemic rats (received 4 g/kg of glucose, po); and diabetic rats.[15]

Diabetic rats in the treated group received **1** (100 mg/kg) by oral gavage or insulin (3.5 nM; 0.5 IU) by ip route. Also, treated

hyperglycemic rats received insulin by ip route.[16] After 24 h, soleus muscles were removed and immediately used for the assay of glycogen content.

Determination of Serum Glucose. Blood samples from the tail vein were collected and centrifuged, and the serum was used to determine glycemia by a glucose oxidase method. For diabetic rats, a serum glucose range of 22–29 mmol/L was used for the experiment.[13,15]

Studies on ^{14}C-Glucose Uptake in the Rat Soleus Muscle. For the ^{14}C-DG uptake experiments, muscles from normal rats were used; one muscle (alternately left and right) from each rat was used as experimental and the contralateral one was used as the control. The muscles were dissected, weighed, and preincubated and incubated at 37 °C in Krebs Ringer-bicarbonate (KRb) buffer of the composition 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, and 25 mM $NaHCO_3$ and bubbled with O_2/CO_2 (95%:5%, v/v) up to pH 7.4. Compound **1** (0.0001, 0.001, 0.1, 1, 10, 20, 200, 520, 1040, 1580, 2080 μM) and insulin (0.007, 0.07, 0.7, 3.5, 7, 35, 70 nM) were added to the preincubation (30 min) and incubation medium (60 min) in the presence or absence of 10 μM LY294002, 40 μM RO318220, 50 μM PD98059, or 0.35 mM cycloheximide. Then ^{14}C-DG (0.1 μCi/mL) was added to each sample during the incubation period. After incubation, the muscles were removed to screw cap tubes containing 1 mL of distilled water. These were frozen at −20 °C in a freezer and boiled afterward for 10 min; 25 μL aliquots of tissue and external medium were placed in liquid scintillation in an LKB rack, beta liquid scintillation spectrometer (model 1215; EG and G-Wallac, Turku, Finland), for the radioactivity measurements. The results were expressed as the tissue/medium (T/M) ratio: cpm/mL tissue fluid per cpm/mL incubation medium and percentage of control.[13]

Glycogen Content Measurements. Glycogen was isolated from the rat soleus muscle as described by Krisman with minor modifications.[23] The tissues were weighed, homogenized in 33% KOH, and boiled at 100 °C for 20 min, with occasional stirring. After cooling, 96% ethanol was added to the samples, which were heated to boiling followed by cooling in an ice bath to aid the precipitation of glycogen. The homogenates were centrifuged at 3,000 rpm for 15 min, the supernatant was discarded, and pellets were neutralized with saturated NH_4Cl, heated to 100 °C for 5 min, washed, and resolubilized in water. Glycogen content was determined by treatment with iodine reagent, and the absorbance was measured at 460 nm. The results were expressed as mg of glycogen/g of tissue.

Statistical Analysis. The results are means ± SEM expressed as the tissue/medium ratio: cpm/mL tissue fluid per cpm/mL incubation medium or % of control. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by a Bonferroni test. Differences were considered to be significant when $p \leq 0.05$.

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Supporting Information Available: Scheme of the action of kaempferol 3-neohesperidoside (**1**) on ^{14}C-glucose uptake in rat soleus muscle. This information is available free of charge via the Internet at http://pubs.acs.org.

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