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Improvement of the circulatory function partially accounts for the neuroprotective action of the phytoestrogen genistein in experimental ischemic stroke

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ABSTRACT

We tested the hypothesis that the phytoestrogen genistein protects the brain against ischemic stroke by improving the circulatory function in terms of reduced production of thromboxane A_2 and leukocyte–platelet aggregates, and of preserved vascular reactivity. Ischemia-reperfusion (90 min-3 days, intraluminal filament) was induced in male Wistar rats, and functional score and cerebral infarct volume were the end points examined. Genistein (10 mg/kg/day) or vehicle (β-cyclodextrin) was administered at 30 min after ischemia or sham-operation. Production of thromboxane A_2 and leukocyte–platelet aggregates, as well as reactivity of carotid artery to U-46619 (thromboxane A_2 analogue) and to platelet releasate was measured. At 3 days post-ischemia, both improvement in the functional examination and reduction in the total infarct volume were shown in the ischemic genistein-treated group. Genistein significantly reverted both the increased thromboxane A_2 concentration and the increased leukocyte–platelet aggregates production found in samples from the ischemic vehicle-treated group. Both U-46619 and platelet releasate elicited contractions of the carotid artery, which were significantly lower in the ischemic vehicle-treated group. Genistein significantly restored both the decreased U-46619- and the decreased platelet releasate-elicited contractile responses. In conclusion, genistein protects the brain against an ischemia-reperfusion challenge, at least in part, by its beneficial effects on the circulatory function.

1. Introduction

About one million strokes occur each year in the European Union, and the total incidence is projected to increase over the next two decades. Stroke is the second cause of death after coronary heart disease and, may be more importantly, the first cause of chronic disability in the European Union (Meairs et al., 2007). In spite of the myriad of potential targets identified during the study of the pathophysiology of stroke, only thrombolysis with the tissue-plasminogen activator has been approved by the American Food and Drug Administration. Unfortunately, its use is restricted to a low percentage of patients and, moreover, it can entail serious detrimental effects (neurotoxicity, reperfusión injury) (Stankowski and Gupta, 2011), so that the need for effective neuroprotective agents still remains.

Platelets play a crucial role in the atherothrombotic events leading to stroke, and changes in platelet function have been reported to occur in the acute phase of ischemia (Franks et al., 2010). Antiplatelet drugs, particularly aspirin, are used nowadays to treat acute arterial thrombotic events. Aspirin inhibits cyclooxygenase-1 and subsequent synthesis of thromboxane A_2. This eicosanoid contributes to the platelet recruitment into the forming thrombus as we reported in aspirin-treated healthy subjects (Valles et al., 1991), as well as in patients with vascular diseases (Santos et al., 2008). However, conventional antiplatelet treatments reduce the risk of serious vascular events only by about 25% (Antithrombotic Trialists' Collaboration, 2002). Therefore, a new generation of safer and more effective antithrombotic drugs is needed. A promising alternative is soy-derived isoflavones, a group of chemicals structurally and functionally related to the ovarian hormone 17β-estradiol (hence the term "phytoestrogens"), which have been repeatedly shown to inhibit platelet

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aggregation (Guerrero et al., 2005; Kondo et al., 2002; Muñoz et al., 2009; Navarro-Núñez et al., 2009).

Isoflavones, particularly genistein, are being increasingly investigated as potential neuroprotectors in ischemic stroke. Others and we have demonstrated that soy-based high-isoflavone diets as well as genistein administered as pure aglycone, reduced cerebral infarct size in rat models of ischemic stroke (Burguete et al., 2006; Castelló-Ruiz et al., 2011; Lovekamp-Swan et al., 2007; Schreihofer et al., 2005; Trieu and Uckun, 1999). In humans, high isoflavone intake has been reported to be associated with reduced cerebral infarction in Japanese women (Kokubo et al., 2007). Evidence obtained in cultured neurons points to the antioxidant and the antiapoptotic action as the main mechanisms mediating the neuroprotective effects of genistein (Arce et al., 2010; Schreihofer and Redmond, 2009; Sonee et al., 2004). Interestingly, the possible involvement of vascular function in the neuroprotective action of genistein in ischemic stroke has not been examined, even though it has been reported to relax cerebral arteries (Torregrosa et al., 2003) and to restore vasodilation and cerebral blood flow autoregulation after brain injury (Hong et al., 2001).

The present study was carried out to test the hypothesis that the neuroprotective effects of genistein in ischemic stroke are mediated, at least partially, by its beneficial effects on platelets and vascular wall as assessed by the antiplatelet effect and the preservation of vascular reactivity, respectively.

2. Materials and methods

2.1. Animal model of ischemic stroke

Experiments were conducted in compliance with the Spanish legislation on "Protection of Animals used for Experimental and other Scientific Purposes", and in accordance with the Directives of the European Community on this subject. Thirty male Wistar rats (300–350 g, Charles River) were housed under standard environmental conditions, and fed an isoflavone-free chow (TD96155 diet, Harlan Teklad) with water ad libitum.

Transient occlusion of the middle cerebral artery (MCAO) was performed by following the intraluminal suture procedure as originally described (Longa et al., 1989), and adapted to our experimental setup (Burguete et al., 2006). This includes continuous monitorization under anesthesia of cerebrocortical laser-Doppler flow (cortical perfusion, CP, laser-Doppler flowmeter PF4001 Master, Perimed, Järfälla, Sweden), arterial blood pressure (ABP, femoral artery catheter connected to a 1290C pressure transducer, Hewlett-Packard, Palo Alto, California, USA, connected to a blood pressure monitor, Stoelting, Kiel, Wisconsin, USA) and core temperature (T, rectal probe YSI 402, Yellow Spring Instruments, Yellow Springs, Ohio, USA, connected to a thermometer and a feedback-regulated heating blanket (HB101/2, Letica, Barcelona, Spain), and discontinuous measurement (femoral artery catheter) of pH, PaO_2, $PaCO_2$ (ABL5, Radiometer España, San Fernando de Henares, Madrid) and glucose (glucometer Ascensia Elite, Bayer Diagnostics, Dublin, Ireland) at the three stages during surgery (pre-ischemia, ischemia and reperfusion). To achieve MCAO, a 3 cm length of 4–0 monofilament nylon suture was gently advanced into the internal carotid artery and circle of Willis until the origin of the MCA was reached. This was indicated by both slight resistance to nylon suture advance and sudden fall in CP. Occlusion was maintained for 90 min, and the filament was carefully retracted afterwards to allow reperfusion, which was monitorized for 30 min. A group of sham-operated animals followed the surgical procedure except the MCAO. At 30 min after MCAO or sham-operation, a single dose of 10 mg/kg genistein (aglycone) or vehicle (β-cyclodextrin 45%) (Sigma-Aldrich) was administered subcutaneously. After the injection, an osmotic pump (2ML1, Alzet, Durect Corp., Cupertino, California, USA) was placed intraperitoneally delivering 10 μl/h genistein, so that the animal received a total dose of 10 mg/kg/day genistein throughout the 3 days post-ischemia. A group of animals received only the vehicle. Therefore, three experimental groups were established: sham-operated vehicle-treated group, MCAO vehicle-treated group, and MCAO 10 mg/kg/day genistein-treated group.

At 3 days reperfusion, four tests were followed to assess the severity of functional deficits induced by ischemia-reperfusion: (a) spontaneous activity, (b) circling to the left, (c) parachute reflex and (d) resistance to left forepaw stretching. Total score could range from 0 (no neurological deficits) to 9 (highest neurological deficits) (Burguete et al., 2006).

To measure cerebral infarct volume rats were exanguinated by aortic cannulation under anesthesia. The whole brain was removed and infarct volume was determined by the 2,3,5-triphenyltetrazolium (TTC) chloride vital staining method (Bederson et al., 1986), followed by morphometric analysis. Briefly, seven 2-mm thick coronal sections were obtained by means of a tissue slicer (Stoelting, Wood Dale, Illinois, USA). Brain slices were immersed in a 2% solution of TTC in saline solution at 37 °C for 15 min, after which slices were fixed in 10% phosphate-buffered formalin (pH 7.4) overnight. Since macrophage infiltration occurs during maturation of the infarct, we obtained an accurate index of brain injury area 72 h after the transient ischemic insult by shortening TTC incubation time to half the originally described time (Bederson et al., 1986), in order to maintain sharp contrast between damaged and healthy tissue. Digital photographs were taken and the border between infarcted and noninfarcted tissue was outlined with an image analysis system (Scion Image v4.02, Scion Corporation, Frederick, Maryland, USA). In order to correct the influence of edema, infarcted area was calculated as follows: corrected infarct area = infarct area *x* [contralateral hemisphere area/ipsilateral hemisphere area]. Corrected infarct area was measured on both the anterior and posterior sides of each slice and averaged; the corrected infarct volume was calculated by multiplying the average corrected infarct area by thickness of the slice (2 mm), and the total corrected infarct volume by adding up the corrected infarct volume of the seven slices. The operational sequence was applied separately to cortex and subcortical regions by researchers who were blinded to treatment group.

2.2. Assessment of platelet function

Thromboxane B_2, the stable metabolite of thromboxane A_2, was determined by enzymoimmunoassay as previously described (Santos et al., 2008). Blood was collected at the time of euthanization into heparinized tubes and platelet-rich plasma and platelet-poor plasma were prepared by differential centrifugation (200 and 2,500*g* respectively, 10 min, 22 °C). To measure arachidonic acid-induced platelet aggregation and thromboxane A_2 synthesis, arachidonic acid (0.5 mol/l) was added to 190 μl of platelet-rich plasma diluted with 110 μl of phosphate-buffered saline in a siliconized aggregometer cuvette under stirring conditions (1,000 *g*, 5 min, 37 °C). After 5 min, indometacin (15 μg/ml) was added to stop the reaction and the sample was centrifuged (10,000 g, 1 min). The cell-free supernatant was collected and kept at −80 °C until thromboxane B_2 measurement (GE Healthcare, Barcelona, Spain).

Leukocyte–platelet aggregates production was measured by flow cytometry (Coulter Epics XL-MCL, Beckman Coulter, Izasa, Barcelona, Spain) as follows. Whole blood was diluted 1:10 with Ca^{2+}-free 20 mmol/l HEPES containing (mmol/l): $MgSO_4$ 0.8, KCl 5.36, KH_2PO_4 0.44, NaCl 137, Na_2HPO_4 0.34, glucose 5.55, prior to labeling with CD45-FITC and CD61-PE for 30 min at room