Chronic Treatment with Red Wine Polyphenol Compounds Mediates Neuroprotection in a Rat Model of Ischemic Cerebral Stroke

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Abstract

In this study, we investigated the in vivo effects of red wine polyphenol compounds (RWPC) in rats that were submitted to middle cerebral occlusion as an experimental model of stroke. Male Wistar rats were given RWPC [30 mg/(kg · d) dissolved in drinking water] or water for 1 wk before being subjected to transient middle cerebral artery occlusion followed by reperfusion. Sham-operated rats were subjected to transient occlusion in which the filament was not completely introduced. The release of amino acids and energy metabolites were monitored by intracerebral microdialysis. The volume of the ischemic lesion was assessed 24 h after reperfusion. Proteomic analysis of brain tissue was performed to study the effects of ischemia and RWPC on specific protein expression. Treatment with RWPC completely prevented the burst of excitatory amino acids that occurred in response to ischemia in untreated rats and significantly reduced brain infarct volumes. Rats chronically treated with RWPC, however, had lower basal concentrations of energy metabolites, including glucose and lactate in the brain parenchyma, compared with untreated rats. Chronic RWPC treatment significantly enhanced the residual cerebral blood flow during occlusion and reperfusion in rats subjected to transient occlusion compared with untreated rats. This effect resulted from arterial vasodilatation, as the internal diameters of several arteries were significantly enlarged after RWPC treatment. Proteomic studies revealed the modulation by RWPC of the expression of proteins involved in the maintenance of neuronal caliber and axon formation, in the protection against oxidative stress, and in energy metabolism. These findings provide an experimental basis for the beneficial effects of RWPC on the neurovascular unit during stroke. J. Nutr. 138: 519–525, 2008.

Introduction

Stroke is a major cause of death in western countries. Ischemic stroke represents the major form of stroke and is characterized by an occlusion of cerebral arteries, leading to a complex cascade of pathophysiological events that damage the brain tissue (1). Increasing evidence suggests that the view of stroke should be integrative with a concept of dynamic interaction among cells belonging to the neurovascular unit, such as endothelial cells, astrocytes, and neurons. The functionality of this unit is altered by the complex series of interconnected pathophysiological processes that damage the brain tissue during stroke as a consequence of the reduction of blood flow that induces an important depletion of energy metabolites, such as glucose and ATP. Few treatments are available today to improve stroke outcome and to restore brain functions. The current therapy uses the administration of a recombinant tissue-type plasminogen activator, which has to be given within 3 h of stroke onset. Nevertheless, the risk of inducing an intracerebral hemorrhage is increased by 6% (2) and the administration of a tissue-type plasminogen activator can lead to a reocclusion in 34% of the cases (3) [for review, Lo et al. (4)]. A combination of agents, including thrombolytic and neuroprotective molecules is needed to fight against this disease.

Epidemiological studies have suggested that diets rich in red wine polyphenol compounds (RWPC) might reduce the risk of cardiovascular diseases (5). Indeed, these compounds possess a

Abbreviations used: 2D, two-dimensional; EAA, excitatory amino acids; ECA, external carotid artery; eNOS, endothelial nitric oxide synthase; Hsp60, Heat shock protein 60; ICA, internal carotid artery; IPG, immobilized pH gradient; MAP-2, microtubule-associated protein-2; MCAO, middle cerebral artery occlusion; NF-M, neurofilament triplet M protein; NO, nitric oxide; RWPC, red wine polyphenol compounds.
number of biological effects that might participate in the protection of the neurovascular unit, including antiaggregatory platelet activity, antioxidant, and free radical scavenging properties. Another therapeutically relevant effect of RWPC may be their ability to interact with the generation of nitric oxide (NO) from vascular endothelium, which leads not only to vasodilatation but also to the expression of genes that are protective for the cardiovascular system. Also, RWPC contribute to the preservation of the integrity of cells belonging to the neurovascular unit by acting on the signaling cascades implicated in apoptosis. In this context, we provided evidence that delphinidin, an anthocyanin contained in RWPC, protects endothelial cells against apoptosis by a mechanism involving NO (6,7). Due to their antioxidant properties, diets supplemented with RWPC might also protect the brain against ischemic damage. RWPC reduce oxidative and nitrosative stresses and lead to the inhibition of neural firings that lead to neuronal death.

We investigated the potential neuroprotective effects of RWPC in a rat model of transient cerebral ischemia, performed by occlusion of the middle cerebral artery, followed by reperfusion (8). This model mimics the clinical situation in patients with embolic stroke. In this study, we reported that RWPC are able to inhibit ischemia-induced excitotoxicity and improve cerebral blood flow and vascular remodeling. We also characterized new potential targets of RWPC on stroke, such as the proteins involved in the maintenance of neuronal caliber and axon formation, protection against oxidative stress, survival of cells, and energy metabolism by differential proteomic approaches, using 2-dimensional (2D) PAGE.

Materials and Methods

Animal experiments. All experiments were approved by the Animal Welfare Committee of the Canton Basel. Male adult Wistar rats (250–350 g, RCC) received tap water or 30 mg/kg - d) RWPC (Provinols, SDF, containing (in g/kg of dry powder): 480 proanthocyanidins, 61 total anthocyanins, 19 free anthocyanins, 38 catechin, 18 hydroxycinnamic acids, 14 flavonols, and 370 polymeric tannins) dissolved in drinking water for 1 wk. Rats were anesthetized with pentobarbital (Nembutal, 50 mg/kg, intraperitoneally) during the entire experimental procedure. Their body temperatures were maintained between 36.5 and 37.5°C with a controlled heating pad.

Intracerebral microdialysis. The anesthetized rats were fixed in a stereotaxic frame and a craniotomy was made using a high-speed dental drill. After the dura-arachnoid membrane had been incised, the microdialysis probe, the right common carotid artery, external carotid artery and the middle cerebral artery occlusion model.

Doppler analysis. Cerebral blood flow was monitored in rats subjected to craniotomy, before, during, and after occlusion with a laser Doppler flowmeter (Perimed AB). The values recorded 10 min before the beginning of the middle cerebral artery occlusion (MCAO) were considered as 100% of the cerebral blood flow.

Measurement of the internal diameter of arteries. Controls and RWPC-treated rats were killed with CO2 placed in the supine position, and the thorax was opened. They were perfused with 5 mL of methylmethacrylate mixture (Technovit 7001 kit) through the ascending aorta, maintained at room temperature for 1 h until resin polymerization was complete, and then macerated in 40% potassium hydroxide solution at 62°C for 24 h. Measurements of the internal diameter of cerebral arteries were made with a digital slide caliper.

Microdialysis. Glucose and lactate concentrations were measured in 12.5 μL of microdialysis sample with an automatic analyzer (YSI 2300 STAT, Yellow Springs Instruments). Amino acids were analyzed by HPLC after automatic precolumn derivatization with o-phthalaldialdeyde, as previously described (9). Free radical scavengers were quantified using a reversed-phase HPLC with electrochemical detection at a gold electrode set at 0.65 mV, as previously described (10). Data were analyzed using the Chromeleon software (Dionex AG). Infarct volume. Brains were removed 24 h after the start of reperfusion, immediately frozen, and sectioned in 20-μm coronal slices with a cryostat. The lesions were visualized by immunohistological staining using anti-microtubule-associated protein-2 (MAP-2) antibodies. The volumes of the MAP-2 negative areas (corresponding to the lesions) were determined using a computer-assisted image analyzer (AnalySIS, Soft Imaging System), as previously described (9).

Extraction of intracellular proteins. Intracellular proteins were extracted from the rat brains at 4°C. Left and right cerebral hemispheres were separated in liquid nitrogen and solubilized in a lysis buffer (CHAPS 4%, urea 7 mol/L, thiourea 2 mol/L, Tris-HCl 50 mmol/L, pH 8.0, 1 protease inhibitor-cocktail tablet (Complete, Roche Diagnostics) for 10 mL buffer). Samples were homogenized using a Polytron for 10 s and centrifuged at 22,000 × g for 15 min. Intracellular proteins were recovered from supernatants and diluted in the lysis buffer at a concentration of 10 g/L and stored at −20°C.

2D gel electrophoresis. 2D gels were performed using 100 μg of proteins as previously described (11). Briefly, proteins were mixed with a loading buffer for IPG strips (Genomic Solutions) and applied in gel for reswelling a dry immobilized pH gradient (IPG) 180-mm, pH 3–10 linear gradient (Amersham Biosciences) on a protein IEF Cell System (Bio-Rad). Isoelectrofocialization was completed in 30 h with the current limited to 50 mA per strip. The equilibrated IPG strips were transferred to the second dimension SDS-PAGE 12% Duracryl gels (170 × 200 × 1.5 mm; Genomic Solutions) and electrophoresis was carried out using the Protean II xi 2-D system (Bio-Rad) with a running buffer (25 mmol/L Tris pH 7.4, 192 mmol/L glycine) containing 0.1% SDS at 15 mA per gel for 16 h. The gels were stained with Coomassie Brilliant Blue R-250 and destained with 20% methanol and 10% acetic acid. Protein spots were quantified using the image analysis software (Image Master Platinum, GE Healthcare). Silver stained–2D gels were digitized at 300 densities per inch resolution and standardized using Image Master Platinum for analysis (GE Healthcare).

The middle cerebral artery occlusion model. During equilibration of the microdialysis probe, the right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline cervical skin incision. The common carotid artery and ECA were permanently cauterized. A 2.2-cm-long 3–0 monofilament suture was introduced at the bifurcation of the ECA and ICA and advanced in the ICA until resistance was encountered. The middle cerebral artery was occluded for 90 min, then the suture was partly withdrawn for reperfusion. A total of 5 sham-operated, untreated rats, in which the filament was not totally introduced, were analyzed to follow the normal time course of variations of certain microdialysis values. Data from these rats were included in statistical analysis when values were determined.
absolute deviation as the dispersion (100%). The quantification of expression was expressed as percent volume where %V = spot volume/volumes of all spots resolved in the gel as described (12). Variations in abundance were calculated as the ratio of mean values (%V) for a spot between 2 groups compared.

Usually, we observed that in global analysis, fold variations in protein are modest by comparison to transcriptomic analysis, as described in the literature (13,14).

Mass spectrometry. To identify the protein spots on the gel, gels were destained using potassium ferricyanide solution and 1.6% sodium thiosulfate, then washed with distilled water. The gel pieces were prepared for trypsin digestion as previously described (15). After washing with 50 μL acetonitrile (ACN), the gel pieces were dehydrated and then rehydrated with 20 μL of 50 mmol/L NH₄HCO₃, pH 8, containing 20 μg/L trypsin (Promega), and the proteins were digested at 37°C overnight. The samples were desalted with Zip Tip C18 (Millipore) and eluted with matrix solution (α-cyano-4-hydroxycinnamic acid) at 10 μg/L in 60% ACN containing 0.01% trifluoroacetic acid. The samples were spotted onto the MALDI-TOF MS target and the dried spots analyzed.

Using a Voyager-DE STR mass spectrometer (PerSeptive Biosystems), the resulting spectra were internally calibrated using the DataExplorer software (PerSeptive Biosystems) with trypsin autoproteolysis products. Products were identified by peptide mass fingerprinting, using ProFound (16) and MS-FIT (17) software against NCBI and UniProtKB/Swissprot databases with the following parameters: rat species, 1 missed cleavage site, and a mass tolerance setting of 50 ppm. Partial chemical modifications, such as carbamidomethylation of cysteine and the oxidation of methionine, were taken into consideration for the queries. The criteria used to accept identifications included the extent of sequence coverage, the number of peptides matched (minimum of 4), the probability score (minimum of 1.645 for the Z-score in Profound; minimum of 70 for the Mowse score for MS-FIT), the mass and pl accuracy, and whether rat protein appeared as the top candidates in the first pass search, where no restriction was applied to the species of origin.

Statistical analysis. All data are expressed as mean ± SEM. Student’s unpaired t test was performed for single comparisons among groups. Microdialysis and laser Doppler data were assessed by the 2-way ANOVA with repeated measures to test the time effect. When significant differences were found, ANOVA was followed by the Bonferroni post hoc test. Statistical significance was accepted at P < 0.05.

Results

Excitatory amino acids. Aspartate and glutamate concentrations were monitored in the cerebral cortex by microdialysis in rats subjected to MCAO and reperfusion. In sham-operated rats, the levels of both amino acids remained stable. A rapid increase in the aspartate concentration occurred immediately after occlusion in control rats (Fig. 1A). It reached a maximum after 30 min of occlusion and then decreased to the basal value during reperfusion. Treatment with RWPC did not affect the basal level of aspartate but completely prevented its release induced by MCAO. All the concentrations measured during occlusion and reperfusion were significantly lower in RWPC-treated rats than in controls. In control rats, the glutamate concentration was also dramatically increased after the onset of occlusion (Fig. 1B) and decreased again during reperfusion. Surprisingly, treatment with RWPC significantly reduced the basal level of glutamate compared with sham-operated rats, and the levels of glutamate in RWPC-treated rats were significantly lower than in controls during occlusion as well as during reperfusion.

A transient peak of taurine was obtained after the onset of occlusion in control rats, with the maximum concentration being reached within 1 h (Fig. 1C). RWPC treatment did not affect the basal level of taurine. A small peak of taurine was obtained during occlusion, but the levels were significantly lower than those measured in controls during MCAO and reperfusion.

Energy metabolism and free radical scavengers. Stroke is associated with reduced delivery of oxygen and glucose. Lactate is produced under hypoxic conditions as a result of glycolysis in astrocytes. We monitored extracellular concentrations of glucose and lactate during MCAO and reperfusion. Although the glucose concentrations decreased slowly in sham-operated rats, MCAO induced a dramatic decrease of the glucose level and reperfusion partially restored this level in controls (Fig. 2A). Chronic treatment with RWPC significantly decreased the basal concentration of glucose measured before MCAO. Occlusion in RWPC-treated rats produced a modest diminution of glucose concentration that did not change further during reperfusion.
Glucose concentrations did not differ between control and RWPC-treated groups after the onset of occlusion and during reperfusion. The glucose levels in the control and RWPC-treated groups remained significantly lower than those of sham-operated rats.

The lactate concentration increased immediately after MCAO in controls (Fig. 2B). Compared with controls, RWPC treatment significantly decreased the level of lactate during occlusion and reperfusion. In both groups, lactate concentrations were significantly higher than in sham-operated rats. Compared with controls, in which ascorbic acid increased during occlusion, RWPC significantly reduced the release of ascorbic acid during occlusion and the beginning of reperfusion (Fig. 2C). RWPC treatment did not modify the basal level and the increase of uric acid observed in controls during occlusion and early reperfusion. However, RWPC induced a significant decrease of uric acid after 2 h of reperfusion (Fig. 2D).

**Cerebral blood flow and infarct size.** Occlusion induced a significant reduction of cerebral blood flow in the controls and RWPC-treated rats. However, the residual cortical blood flow measured during occlusion was significantly higher in RWPC-treated rats compared with untreated rats (Fig. 3A). Furthermore, RWPC significantly enhanced blood flow after reperfusion, compared with controls. To better understand the mechanisms of action of RWPC on cerebral arteries, we measured the internal diameters of various arteries (indicated in Fig. 3B). The internal diameters of arteries from the controls were significantly smaller than those of RWPC-treated rats (Fig. 3C), suggesting an outward remodeling of the cerebral blood vessels.

The volumes of the lesions were measured 24 h after the start of reperfusion using MAP-2 immunohistochemistry. The lesions measured in the control rats were localized mainly in the cortex and the striatum. In the rats treated with RWPC, lesions were seen only in 2 of 7 rats and were significantly smaller than in the controls (control rats, 358 ± 49 mm³; RWPC-treated rats, 125 ± 46 mm³; P < 0.05). The ischemic hemispheres from the 5 remaining rats showed only signs of edema, without lesion (data not shown).

**Proteomic analysis.** To get further insight into the molecular mechanisms of the beneficial effect by RWPC on cerebral ischemia, we developed a proteomic approach to identify proteins differentially expressed in the rat brain. We analyzed the isolated proteins by 2D gel electrophoresis to produce high-resolution protein maps of the left (nonischemic) and right (ischemic) hemispheres from control and RWPC-treated rats (Supplemental Fig. 1A). In left and right hemispheres, we detected 569 ± 6245 and 765 ± 119 spots, respectively, in control rats, and 818 ± 102 and 549 ± 124 spots, respectively, in RWPC-treated rats. Computer-based 2D gel analysis identified 25 polypeptidic spots differentially expressed from all bioinformatic analysis performed (Supplemental Fig. 1B). In detail, when comparing left and right hemispheres of control rats, computer-based 2D gel analysis detected 374 matched spots from which 19 were differentially expressed, 5 were upregulated and 14 downregulated. When comparing left and right hemispheres of RWPC-treated rats, 302 matched spots were detected and 14 of them were differentially expressed, 5 upregulated and 9 downregulated. The comparison of the left hemispheres of the control and RWPC-treated rats detected 310 matched spots, 15 spots differentially expressed, 8 upregulated and 7 downregulated. Among right hemispheres of the control and RWPC-treated rats, 18 spots were differentially expressed, 11 were upregulated and 7 downregulated.

Among the 25 polypeptidic spots identified by MALDI-TOF (Supplemental Table 1), several proteins identified were brain-specific, like neurofilament triplet M protein (NF-M), calreticulin, fructose-biphosphate aldolase C, TOAD-64, 14–3–3 protein...
observed that treatment with RWPC decreased the expression of 3 proteins not affected by ischemia: UCHL-1, RhoGDP-dissociation inhibitor, and Tpi protein. Interestingly, for 14–3-3 protein β/α and proteasome β type 6, the decrease of expression induced by ischemia was strengthened in RWPC-treated animals.

Regarding the effect of RWPC on ischemic and nonischemic brain (Table 2), we observed a decreased expression in the ischemic brain for proteins such as albumin, TOAD-64, and Heat shock protein 60 (Hsp 60). As for the effect of ischemia, we observed a strengthened modulation of expression for 14–3-3 protein β/α and proteasome β type 6 with RWPC. The opposite effect was observed for pyruvate kinase isozymes M1/M2, whose expression was decreased in the nonischemic hemisphere and increased in the ischemic hemisphere, and Tpi protein, whose expression was observed no variation in the ischemic hemisphere but a modulation in the nonischemic hemisphere.

Interestingly, for several proteins, we observed no variation in nonischemic brain but a modulation in the ischemic brain, like for aconitase hydratase, fructose-biphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate mutase 1; and 3 were involved in the Krebs cycle: voltage-dependent anion-selective channel protein 1, α-tropomyosin 3, and ubiquitin carboxy-terminal hydrolase isozyme L1.

### Discussion

The new neuroprotective strategies against stroke target the preservation of endothelium integrity and the deleterious effects induced by reactive oxygen species. We took advantage of the pleiotropic effects of natural dietary polyphenols, especially RWPC, as potential neuroprotective drugs against stroke. The dose of RWPC used (30 mg/(kg · d)) corresponds to high consumption of red wine in terms of volume and therefore the beneficial effects of polyphenols can be blunted with the amount of ethanol in this beverage. We used RWPC without alcohol in this study, and
TABLE 2  Variations in protein expression in ischemic and nonischemic hemispheres of rats treated with RWPC

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Fold variation in brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonischemic</td>
</tr>
<tr>
<td>NF-M</td>
<td>1.96</td>
</tr>
<tr>
<td>Acotinase hydratase</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>1.71</td>
</tr>
<tr>
<td>TOAD-64</td>
<td>1.68</td>
</tr>
<tr>
<td>60 kDa heat shock protein</td>
<td>2.31</td>
</tr>
<tr>
<td>Pyruvate kinase isozymes M1/M2</td>
<td>–1.20</td>
</tr>
<tr>
<td>α-enolase</td>
<td>–1.18</td>
</tr>
<tr>
<td>Fructose-biphosphate aldolase A</td>
<td>NV</td>
</tr>
<tr>
<td>Fructose-biphosphate aldolase C</td>
<td>NV</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>–1.20</td>
</tr>
<tr>
<td>Malate dehydrogenase 1</td>
<td>–1.29</td>
</tr>
<tr>
<td>Malate dehydrogenase 2</td>
<td>1.77</td>
</tr>
<tr>
<td>Voltage-dependent anion-selective</td>
<td>–1.11</td>
</tr>
<tr>
<td>channel protein 1</td>
<td></td>
</tr>
<tr>
<td>α-tropomyosin 3</td>
<td>NA</td>
</tr>
<tr>
<td>Phosphoglycerate mutase 1</td>
<td>1.13</td>
</tr>
<tr>
<td>14–3–3 protein β/δ</td>
<td>–1.29</td>
</tr>
<tr>
<td>Ubiquitin carboxy-terminal hydrolase isoyme L1</td>
<td>1.12</td>
</tr>
<tr>
<td>Rh GDP dissociation inhibitor 1</td>
<td>NV</td>
</tr>
<tr>
<td>Tpi protein</td>
<td>1.47</td>
</tr>
<tr>
<td>Glutathione S-transferase μ 1</td>
<td>NV</td>
</tr>
<tr>
<td>Proteasome β type 6</td>
<td>–1.74</td>
</tr>
</tbody>
</table>

¹ NA, data not available.
² NV, no variation.

Therefore, RWPC can be used either as complementing human nutrition or as a therapeutic agent to fight against the deleterious effects of stroke. Although RWPC might be of therapeutic benefit in cardiovascular diseases, prospective controlled clinical studies are still lacking. Nevertheless, chronic treatment with RWPC partially restored cerebral blood flow during occlusion and ameliorated the flow during reperfusion in the cortex. This increase was associated with an increase of the internal diameter of the arteries of the cerebral tree, suggesting vasodilatation that enhanced cerebral blood flow. Our previous studies demonstrated that RWPC induce the synthesis and release of NO from the vascular endothelium via an increase in cytosolic calcium (18,19). Furthermore, RWPC possess a long-term effect on NO production by increasing endothelial nitric oxide synthase (eNOS) expression (20), leading to an improvement in endothelial dysfunction in the aorta of hypertensive rats (21,22). Interestingly, RWPC inhibit the expression of the inducible form of NO synthase, which is induced after ischemia and contributes to the late-phase damage. Chronic upregulation of eNOS by RWPC might constitute a preventive approach to reduce tissue injury in patients with a risk of cerebral ischemia.

Moreover, this property of RWPC might improve the outcome after an insult, because NO itself plays a role in different phases of stroke at the level of the neurovascular unit. Indeed, NO can prevent thrombosis, oxidative damage, inflammation, and apoptosis. Apoptosis of endothelial cells strongly affects endothelium permeability and thus facilitates the development of inflammation. We found that delphinidin protects endothelial cells against apoptosis (23) by increasing eNOS expression and release of NO via a MAP kinase inhibitor-sensitive pathway and by decreasing cytochrome c release from mitochondria. Finally, NO can mobilize stem and progenitor cells involved in neovascularization and in the rescue of vascular and neuronal damage. In an ex vivo rat model of cardiac ischemia/reperfusion, short-term RWPC treatment protects against postischemic infarction by decreasing oxidative stress and stimulating a NO-dependent pathway (24). We extend such observations in cerebral ischemia in the MCAO model.

RWPC prevented the occlusion-induced release of excitatory amino acids (EAA) by a mechanism that is not completely understood. RWPC might interfere with the mechanisms leading to glutamate efflux. Glutamate efflux is induced by a calcium-dependent release from vesicles via volume-activated anion channels, across disrupted cell membranes, or through reversed operation of glutamate transporters. Low levels of resveratrol, a polyphenol present in grapes and red wine, has been recently shown to increase glutamate uptake in cortical astrocytes (25), suggesting that RWPC regulate the extracellular concentration of this EAA. Another possibility is that the increased residual blood flow in RWPC-treated rats was enough to prevent the massive release of EAA. However, hypoxia occurred in the middle cerebral artery territory, as indicated by the massive rise of lactate during MCAO in these rats. The reduced release of tau-rine, which is driven in part by the activation of glutamate receptors (26), may be related to the lower excitotoxicity. Surprisingly, RWPC did not significantly modify the profile of the free radical scavengers ascorbic and uric acids, although RWPC modulated intracellular stress proteins and metabolisms sensitive to oxidation, as shown by the proteomic analysis (see below).

Interestingly, chronic treatment with RWPC significantly decreased the basal concentration of glucose in the brain extracellular tissue. This effect might result from the ability of flavonoids to inhibit the intestinal glucose isofrom 2 transporter. This property may therefore reduce glucose absorption (27) and lower the concentration of glucose in the plasma as well as in the brain tissue.

RWPC did not significantly modify the extracellular profiles of the free radical scavengers ascorbic and uric acids compared with untreated ischemic rats. These observations suggest that RWPC preserve the production of free radical scavengers during ischemic conditions. As shown by proteomic analysis, RWPC modulated the expression of intracellular stress proteins and metabolisms sensitive to oxidation (see below). Altogether, RWPC, by reducing disorder of cerebral blood flow, may prevent stroke and may establish early reperfusion during the acute phase of stroke, reducing the magnitude and the extent of tissue injury.

The proteomic analysis gets further insight in proteins modulated by ischemia and RWPC in our model despite the modest variations observed for some proteins. Most of the proteins differentially expressed in stroke play a role in neuroprotection, in the maintenance of neuronal caliber and axon formation and oxidative stress, energy metabolism, and inflammation. For example, NF-M, which is responsible for the control of the neuronal caliber and dendritic arborization and may also protect the cells against oxidative stress (28,29), or TOAD-64, which plays a role in axon formation by acting on the neuronal polarity (30), was differentially expressed by ischemia and/or RWPC.

As published by Lo et al. (4), the major pathways of cerebral ischemia involved oxidative and nitrosative stresses, excitotoxicity, ionic imbalance, apoptosis, and proteins localized in the mitochondria and endoplasmic reticulum. We identified differentially expressed proteins that can be S-nitrosylated, like glyceraldehyde-3-phosphate dehydrogenase. Ischemia and RWPC also modulated the expression of the 14–3–3 protein, a protein kinase C inhibitor involved in cellular differentiation, proliferation, and

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survival (31) and that can form a cluster with proteins involved in glycolysis, like glyceraldehyde 3-phosphate dehydrogenase and Tpi. Interestingly, ischemia induced a decline of enolase 1, a subunit of enolase, interconverting 2-phosphoglycerate to phosphoenolpyruvate during glycolysis (32). Lowering enolase activity resulted in abnormal growth and reduced metabolism in the brain (33). RWPC, however, restored a normal expression of this protein during ischemia. Ubiquitin-positive bodies are the pathological hallmark of many neurodegenerative diseases. The expression of the proteasome β type 6 involved in an ATP/ubiquitin-dependent nonlysosomal proteolytic pathway and in the ubiquitinloylation of RhoA was drastically reduced by RWPC treatment. RWPC also decreased the expression of RhoGDP in the ischemic area, a protein involved in the regulation of small G proteins, such as Rho A, Rho B, and Rac, which control cellular proliferation, differentiation, and apoptosis and regulate NADPH oxidase (34). The expression of Hsp60, a stress-inducible mitochondrial matrix protein was reduced by RWPC in the ischemic brain. In contrast, the expression of VDAC1, a major mitochondrial outer-membrane transporter controlling metabolite traffic, reduced by ischemia, was restored by RWPC.

Altogether, these proteome data highlight potential target pathways modulated by RWPC in the ischemic brain, such as energy metabolism, proteolytic pathways, mitochondrial function, and apoptosis. These data provide an experimental basis for the beneficial effects of RWPC for stroke protection either as prevention or treatment of the different phases of the disease.

Literature Cited