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Quercetin, Kaempferol and Biapigenin from *Hypericum perforatum* are Neuroprotective Against Excitotoxic Insults

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In the present study we investigated the effects of phenolic compounds present in *Hypericum perforatum* against neuronal excitotoxicity and mitochondrial dysfunction. Quercetin, kaemp-ferol and biapigenin significantly reduced neuronal death caused by 100 μM kainate plus 100 μM *N*-methyl-D-aspartate. The observed neuroprotection was correlated with prevention of delayed calcium deregulation and with the maintenance of mitochondrial transmembrane electric potential. The three compounds were able to reduce mitochondrial lipid peroxidation and loss of mitochondrial transmembrane electric potential caused by oxidative stress induced by ADP plus iron. Moreover, biapigenin was also able to significantly affect mitochondrial bioenergetics and decrease the capacity of mitochondria to accumulate calcium. Taken together, the results suggest that the neuroprotective action induced by quercetin and kaempferol are mainly mediated by antioxidant effects, whereas biapigenin mainly affects mitochondrial bioenergetics and calcium uptake.

Keywords: Hypericum perforatum; Excitotoxicity; Neuroprotection; Mitochondrial dysfunction; Calcium homeostasis

Abbreviations

BHT, butylhydroxytoluene; **BSA**, bovine serum albumin; **CsA**, cyclosporin A; **FCCP**, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; **KA**, kainic acid; **MAP-2**, microtubulle associated protein; $\Delta\psi_m$, Mitochondrial transmembrane potential; **NMDA**, *N*-methyl-D-aspartate; **PI**, propidium iodide; **ROS**, reactive oxygen species; **TPP^+**, tetraphenylphosphonium cation; **TBARS**, thiobarbituric acid-reactive species.

INTRODUCTION

Excitotoxicity, resulting from synaptic dysfunction processes, involves excessive glutamate receptor activation and neuronal degeneration. *N*-methyl-D-aspartate (NMDA), 2-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors are ionotropic glutamate receptors responsible for glutamatergic synaptic transmission (Lipton and Rosenberg, 1994; Carafoli *et al.*, 2001; Rego *et al.*, 2001). However, under prolonged NMDA receptor activation, *e.g.*, during ischemia, anoxia, and in other neurodegenerative pathologies (Nicholls and Budd, 1998; Brookes *et al.*, 2004; Nicholls, 2004; Chen and Lipton, 2006), massive

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calcium influx into the cell occurs, leading to failure in neuronal calcium homeostasis (Montal, 1998; Nicholls and Budd, 1998; Nicholls and Ward, 2000; Carafoli *et al.*, 2001; Vergun *et al.*, 2001; Nicholls, 2004; Isaev *et al.*, 2005).

Mitochondria play a central role in calcium homeostasis, but excessive mitochondrial calcium accumulation can also result in loss of mitochondrial transmembrane potential ($\Delta\psi_m$) and uncoupling of respiratory chain, increasing the production of oxygen and nitrogen reactive species. Impairment of mitochondrial function can compromise ATP production and, consequently, lead to depletion of ATP stores and failure of ion homeostasis, including regulation of calcium concentration (Nicholls and Budd, 1998; Nicholls, 2004). Oxidative stress and mitochondrial calcium overload can trigger the opening of the mitochondrial permeability transition pore, which allows the passage of solutes and large molecules into the matrix space (Vieira *et al.*, 2000; Isaev *et al.*, 2005). Consequently, swelling of mitochondria and rupture of the outer mitochondrial membrane occurs leading to loss of mitochondrial function, production of reactive oxygen and nitrogen species causing oxidation of membranes, proteins and nucleic acids. These processes promote the release of pro-apoptotic factors, such as cytochrome c, apoptosis-inducing factor (AIF), Smac-DIABLO and endonuclease G, which trigger the activation of cell death effector mechanisms (Vander Heiden and Thompson, 1999; Bouchier-Hayes *et al.*, 2005). It is now well accepted that mitochondria are central players in the toxic events associated with overactivation of glutamate receptors, with consequent loss of ion homeostasis and downstream pathways ending in neuronal death (Vander Heiden and Thompson, 1999; Nicholls and Ward, 2000; Duchen, 2004; Bouchier-Hayes *et al.*, 2005; Kushnareva *et al.*, 2005;). The critical role of mitochondria in the maintenance of the bioenergetic redox status, together with the participation in cell fate decision, highlight this organelle as a key target for the development of new therapeutic strategies in ischemic injury and neurodegenerative diseases such as Alzheimer's disease (Nicholls and Budd, 1998; Won *et al.*, 2002; Mattson and Kroemer, 2003; Duchen, 2004; Bouchier-Hayes *et al.*, 2005), where excitotoxic events are central players in neuronal death.

Previously, we have shown that *Hypericum perforatum* extracts enriched in flavonoids (Silva *et al.*, 2005) are neuroprotective against β-amyloid-induced toxicity, in primary cultures of rat hippocampal neurons (Silva *et al.*, 2004). *H. perforatum* has also been reported to be effective in an animal model of ischemia and reperfusion injury, reducing physiological and histological signs of damage (De Paola *et al.*, 2005). Accordingly, flavonoids have been reported as potentially effective in preventing cell damage resulting from stroke and ischemia-reperfusion (Dajas *et al.*, 2003; Simonyi *et al.*, 2005; Zhao, 2005). In the present study, we evaluated the neuroprotective properties of phenolic compounds present in *H. perforatum* extracts - quercetin, kaempferol and biapigenin - against excitotoxicity, and further investigated mitochondrial targets potentially involved in the neuroprotective pathways.

EXPERIMENTAL PROCEDURE

Chemicals

Kainate was supplied by Ocean Produce International (USA) and NMDA was supplied by Tocris (USA). Alexa Fluor conjugated antibodies, Calcium Green-5N, Fura2-AM, Hoechst 33342, Mitotracker Red CMXRos, propidium iodide, Syto-13, TMRM were supplied by Molecular Probes (USA). Atractyloside, bongkrekic acid, cyclosporin A, dichlorofluorescein diacetate (DCF), protease (Subtilisin, Carlsberg) type VIII and tetraphenylphosphonium-chloride (TPP) were obtained from Sigma (Spain). Digitonin and pluronic acid were obtained from Calbiochem (USA). Anti-active caspase-3 antibody was obtained from Promega (USA). Anti-active caspase-3 antibody was obtained from Promega (USA). MK-801 was a kind gift of Merck Sharp and Dohme, USA. Quercetin, kaempferol and biapigenin were isolated by preparative HPLC from an *H. perforatum* extract, as described elsewhere (Dias *et al.*, 1998). Purity for all three compounds was 98-99%. All other chemicals were of the highest grade of purity commercially available.

Neuronal Cultures

Hippocampal neurons were dissociated from hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (2.0 mg/ml, 15 min, 37ºC)

and deoxyribonuclease I (0.15 mg/ml) in Ca^{2+} and Mg^{2+} free Hank's balanced solution (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4.2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, supplemented with 0.001% phenol red, 1 mM pyruvate, and 10 mM HEPES, pH 7.4). The cells were cultivated in B-27 supplemented serum-free Neurobasal medium (Gibco), containing glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml), as described previously (Silva *et al.*, 2001). Cultures were kept at 37ºC in a humidified incubator in 5% CO_2/95% air, for 7 days, the time required for maturation of hippocampal neurons. For viability studies with Syto-13 and propidium iodide (PI) cells were plated at a density of 45 x 10^3 cell/cm^2 on poly-D-lysine-coated (0.1 mg/ml) coverslips.

Cell Viability and Immunocytochemical Assays

Neuronal viability was assessed by using the Syto-13 and propidium iodide (PI) live/death assay after exposure of cultured hippocampal neurons to kainate plus NMDA, alone or in the presence of the compounds. The structural formulas of the three compounds tested are shown in figure 1A. Neurons were exposed continuously to 100 μM kainate plus 100 μM NMDA, for 35 minutes at 37ºC, and left to recover for 24 hours in conditioned medium. Syto-13 is a green fluorescent membrane-permeable dye. PI is a non-permeable red fluorescent dye which only stains cells that lost membrane integrity - late apoptotic or necrotic (Silva *et al.*, 2004). Cell death resulting from the isolation procedure and plating accounted for 30% highly condensed PI-positive nuclei (data not shown).

Immunocytochemistry was performed for microtubule-associated protein MAP-2, and mitochondrial morphology and nuclear morphology were evaluated by using MitoTracker Red CMXRos and Hoechst 33342, respectively. Briefly, cells were incubated for 30 min with 125 nM MitoTracker Red CMXRos, washed and fixed with paraformaldehyde (4% paraformaldehyde, 4% sucrose in phosphate buffer) for 30 min. After washing, cells were permeabilized with 0.2% Triton X-100, blocked with 3% bovine serum albumin (BSA) and then incubated with primary antibody mouse anti MAP-2 for 1 hour. After washing, cells were incubated with the conjugated secondary antibody Alexa Fluor 488 Goat Anti-Mouse IgG. After washing, cells were incubated for 5 min with Hoechst 33342 and then mounted in glass coverslips using fluorescence mounting medium (Dako Cytomation, USA).

Calcium Deregulation and Loss of $\Delta\psi_m$ - Single Cell Imaging Assays

Calcium deregulation and mitochondrial transmembrane potential ($\Delta\psi_m$) were monitored by single-cell imaging of cultured hippocampal neurons. Cells were loaded for 40 min with 5 μM Fura-2/AM, 20 nM tetramethylrhodamine methyl ester (TMRM), 0.1% fatty acid free BSA and 0.2% pluronic acid F-127 in Krebs buffer (132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES-Na, pH 7.4). Experiments were carried out at room temperature. The coverslips were rinsed with Krebs buffer and mounted in a perfusion chamber. Image acquisition was performed using the MetaFluor software (Universal Imaging Corporation, ver. 5.0r7, 2003) in an Axiovert 200 epi-fluorescence inverted microscope (Zeiss), equipped with a Lambda DG-4 (Sutter Instrument Company) and a high-resolution LCD-camera (CoolSnap HQ, 100W HBO mercury lamp). Image acquisition was performed alternately at 340, 380 and 598 nm (300 ms exposure time, 10s between acquisitions), using a Fura-2/rhodamine filter. Five minutes after starting image acquisition, cells were exposed to Krebs medium containing 20 nM TMRM and 100 μM kainate plus 100 μM NMDA.

In another set of experiments cells were pre-incubated for 15 min with 10 μM of quercetin, kaempferol or biapigenin (after the loading period and prior to the beginning of the experiment). After this pre-incubation period, the compounds were present for the remaining period of the experiment. Adequate controls were performed using cells perfused with Krebs. Only 5% of the total cells lost calcium homeostasis for the total time of the experiment (40 minutes). The polyphenol concentration was determined after performing a dose-effect study. At 10 μM the three compounds were not toxic and significantly protected neurons from excitotoxic aggression.

Measurement of ROS Generation

Intracellular peroxides were measured as described previously, with minor modifications (Rego *et al.*, 2003), by following the oxidation of dichlorodihydrofluorescein diacetate ($DCFH_2$-DA, 10 μM) to the fluorescent DCF. The neurons were loaded with $DCFH_2$-DA for 30 min in Krebs buffer at 37ºC, in the dark. Basal DCF fluorescence was measured during the first 5 minutes, at which 100 μM kainate plus 100 μM NMDA were added to the cuvette and fluorescence was monitored for the subsequent 35 min. Excitation was set at 502 nm and emission at 550 nm. A SPEX Fluorolog spectrometer (Edison, NJ, USA) equipped with a temperature-controlled water bath and constant stirring was used for these experiments. Loading of the probe was renewed for each experiment. H_2O_2 (10 mM) was used as a positive control.

Mitochondrial Respiratory Chain and $\Delta\psi_m$ Assays

Brain mitochondria were isolated from male Wistar rats (8 weeks old), using a method previously described and used to evaluate $\Delta\psi_m$ and respiration (Moreira *et al.*, 2002). The $\Delta\psi_m$ was monitored by evaluating transmembrane distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP-selective electrode with a calomel electrode as reference (Kamo *et al.*, 1979). The difference in potential between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously. Reactions were carried out at 30ºC in a chamber with magnetic stirring in 1 ml of medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 10 μM EGTA, 5 mM HEPES, pH 7.4, supplemented with 2 μM rotenone) and containing 3 μM TPP-Cl (Moreira *et al.*, 2002; Oliveira *et al.*, 2004). Mitochondria (0.8 mg/ml) were incubated for 3 min with 10 μM quercetin, kaempferol or biapigenin. The reactions were started by adding 8 mM succinate to mitochondria in suspension. After reaching a steady-state distribution of TPP (plateau), 125 μM ADP was added and alterations in $\Delta\psi_m$ recorded. Adequate controls were performed without added compounds, but respecting the same 3 min lag phase used in the test conditions. Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark oxygen electrode connected to a suitable recorder in a 1 ml thermostatic, water-jacketed closed chamber with magnetic stirring (Estabrook, 1967). State 3 respiration is defined as the consumption of oxygen in the presence of substrate and ADP, whereas state 4 respiration is defined as the consumption of oxygen after ADP consumption. Mitochondrial respiration was not altered by the presence of TPP (data not shown). RCR values obtained were in accordance with the expected values for brain mitochondria and previously reported (Moreira *et al.*, 2005).

Mitochondrial Lipid Peroxidation

The extent of lipid peroxidation was directly evaluated in non-energized mitochondria by the formation of thiobarbituric acid reactive species (TBARS) and by respiratory chain-independent oxygen consumption of isolated mitochondria exposed to ADP plus iron; and indirectly by monitoring changes in $\Delta\psi_m$ of energized mitochondria exposed to the oxidant pair ADP plus iron. Briefly, lipid peroxidation of mitochondrial membranes (0.8 mg/ml) was assessed by monitoring oxygen consumption as reported before (Ferreira *et al.*, 1999), with minor modifications, or by evaluating the decay of $\Delta\psi_m$ (mitochondria energized with 8 mM succinate) (Abreu *et al.*, 2000), after exposure to 1 mM ADP plus 100 μM iron for 10 or 15 min at 30ºC. For evaluation of the effect of phenolic compounds, mitochondria were pre-incubated for 3 minutes with 10 μM quercetin, kaempferol or biapigenin. At minute 10 (time point for the end of reaction) samples were taken for evaluation of the extent of lipid peroxidation in non-energized mitochondria by measuring TBARS formation. Butylhydroxytoluene (BHT, 30 μM) was used as a positive control of antioxidant effect against ADP/iron-induced lipid peroxidation.

Mitochondrial Calcium Loading Capacity and Calcium Uptake

Calcium uptake was assessed by following Calcium Green-5N fluorescence with appropriate calibration in the presence of mitochondria (0.2 mg/ml) and rotenone with pulses of 3 μM calcium each. Reactions occurred at 30ºC in a quartz cuvette with 2 ml reaction medium and under magnetic stirring. Changes in fluorescence intensity were monitored using a fluorimeter Perkin Elmer LS 50B (excita-

tion at 506 nm, emission at 531 nm, 5 nm slit) after energization with 4 mM succinate (Oliveira *et al.*, 2003). When steady state fluorescence was achieved, FCCP was added to evaluate calcium release caused by mitochondrial depolarization and, therefore, the amount of calcium that was accumulated in mitochondria due to transmembrane electric potential; calcium uptake into mitochondria assessed with this method was always near 90-95% of the total calcium uptake (data not shown). Calcium uptake was also evaluated upon addition of pulses of 10 μM calcium to energized mitochondria. Pulses were added each 30 s and fluorescence intensity was monitored. The effect of the compounds (pre-incubated for 3 min) on the number of pulses supported by mitochondria before failure in mitochondrial calcium accumulation was also evaluated. Compounds were added after energization with succinate. EGTA was added after achieving final steady-state fluorescence.

Statistical Analysis

Results are presented as means ± SEM of the indicated number of experiments, usually run in triplicate or quadruplicate unless otherwise stated. Statistical significance was determined by using the one-way ANOVA test for multiple comparisons, followed by Bonferroni post-test.

RESULTS

Phenolic Compounds Present in *H. perforatum* are Neuroprotective Against Excitotoxicity in Cultured Hippocampal Neurons

Neuronal viability was assessed after exposure of cultured hippocampal neurons to an acute excitotoxic challenge. Viability significantly decreased after exposure to 100 μM kainate plus 100 μM NMDA (55 ± 1% following excitotoxic insult as compared to 100 ± 4% in the control). Cell death evaluated after 24 h, was significantly prevented in the presence of 10 μM quercetin, kaempferol or biapigenin, with viability of 77 ± 9%, 77 ± 12% and 87 ± 11% of the control, respectively (FIG. 1B). MK-801 (10 μM) blocked kainate plus NMDA-induced neuronal death (FIG. 1B).

We also analyzed changes in morphology as a sign of neurodegeneration. Immunostaining against MAP-2 revealed a marked dendritic degeneration following exposure to kainate plus NMDA. Moreover, we took advantage of MitoTracker Red CMXRos which accumulates in polarized mitochondria, to evaluate changes in mitochondrial distribution in neuronal cells. Following excitotoxic insults a major redistribution of partially depolarized mitochondria was observed with mitochondrial accumulation in cytoplasmatic clusters. Nuclear morphology was assessed by Hoechst 33342 staining, showing highly condensed nuclei in cells exposed to kainate plus NMDA. Exposure to 10 μM biapigenin partially protected neurons from the deleterious action of the excitotoxic aggression, since neurons showed less dendritic dystrophy, maintenance of mitochondrial distribution and reduced number of nuclear markers of cell death (FIG. 1C).

Phenolic Compounds Present in *H. perforatum* Delayed Calcium Deregulation Induced by Kainate plus NMDA

We monitored intracellular calcium concentrations and, simultaneously, $\Delta\psi_m$ by using single-cell imaging of neurons exposed to kainate plus NMDA. A similar protocol to the viability assays was used, aiming to find a correlation between neuroprotection by the phenolic compounds, protection from delayed calcium deregulation and improvement of mitochondrial function. Figure 2A shows representative images of a control (Fura-2 and TMRM imaging) for the critical time points of the experiment: changes in Fura-2 fluorescence (excitation ratio 340/380 nm) are shown in the left panel, whereas the right panel shows TMRM fluorescence. All images were taken from the same field and obtained at different time points, as indicated in the left. Loss of calcium homeostasis (340/380 ratio shifting towards the red color) was strongly correlated with a significant decrease in TMRM fluorescence (fluorescence at 598 nm, shift to black) (r^2=0.96, n=6-8 independent experiments, average 120 cells per field). Following the excitotoxic insult, a significant percentage of cells lost the ability to preserve calcium homeostasis and showed delayed calcium deregulation (FIG. 2 B): top panel shows representative recordings for intracellular calcium concentrations for several control (black) and biapigenin-treated neu-

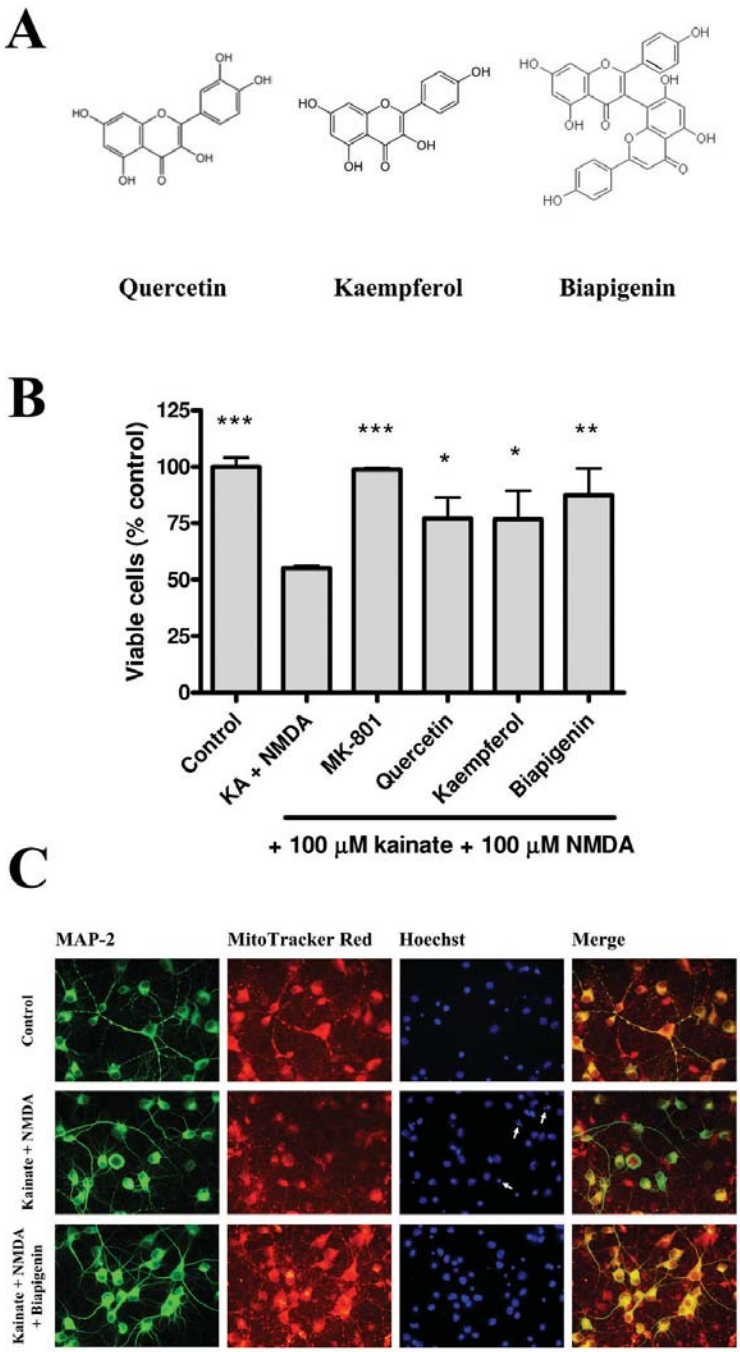

FIGURE 1 **A**) Structural formulas of the phenolic compounds used in the present study: quercetin, kaempferol and biapigenin. **B**) Neuroprotection against excitotoxicity in cultured rat hippocampal neurons. Cell viability was evaluated by the live/death assay Syto-13/PI. Significant neuroprotection against exposure to kainate plus NMDA was afforded by incubation with MK-801, quercetin, kaempferol and biapigenin (10 μM). **C**) Immunocytochemistry for MAP-2, staining with MitoTracker Red CMXRos and nuclear staining with Hoechst 33342. Exposure to kainate plus NMDA induced major changes in neuronal morphology (assessed by microtubule-associated protein MAP-2), in mitochondrial physiology/subcellular distribution (MitoTracker Red CMXRos) and nuclear condensation (arrow). Following excitotoxic insult, signs of dendritic network dystrophy and loss of mitochondrial transmembrane electric potential ($\Delta\psi_m$) were identified. **Top row** - control; **middle row** - kainate plus NMDA; **bottom row** - biapigenin plus kainate plus NMDA. Values are presented as mean ± SEM (n=3 independent experiments); *p <0.05, **p <0.01, ***p <0.001 (comparatively to kainate plus NMDA).

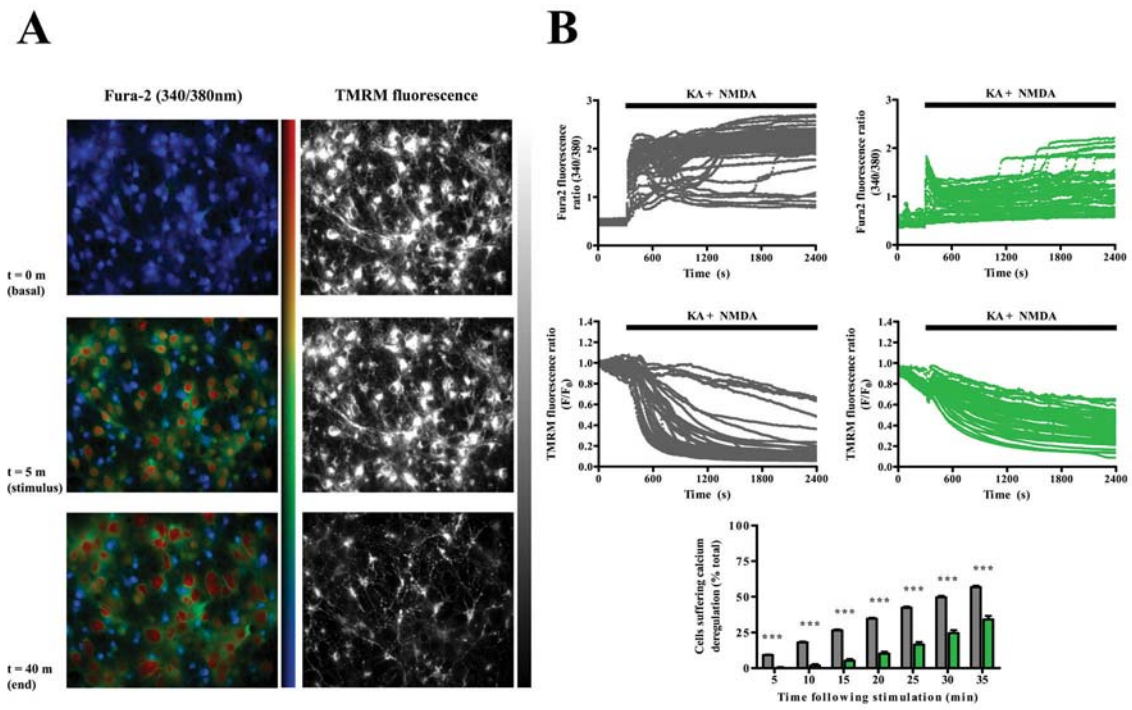

FIGURE 2 Biapigenin significantly protects hippocampal neurons from delayed calcium regulation and failure of mitochondrial potential homeostasis, following excitotoxic insult with kainate plus NMDA. **A**) Calcium deregulation and loss of mitochondrial transmembrane potential ($\Delta\psi_m$) in a typical control experiment, without addition of phenolic compounds. **B**) Addition of 10 μM biapigenin (green) significantly protected neurons from kainate plus NMDA-induced calcium deregulation and loss of mitochondrial membrane potential, when compared with to the control (black). Traces represent control biapigenin-treated cells (**B**, top); the average normalized TMRM fluorescence of the same control (black) and biapigenin-treated (green) (**B**, middle). In **B**) bottom, values were determined for each 5 min and represent the mean of five to eight independent experiments. Time t'=35 min reflects the total number of cells that lost the ability to maintain calcium homeostasis. Scales of fluorescence intensity are indicated at the right of each set of images. Values are presented as mean ± SEM (n=6 to 8 independent experiments); $^{**}p$ <0.01, $^{***}p$ <0.001 (comparatively to kainate plus NMDA).

rons (green), whereas middle panel shows representative recordings of TMRM fluorescence for the same population of neurons and for the same conditions; results are summarized in bottom panel. Exposure of cells to biapigenin (10 μM) significantly protected neurons from delayed calcium deregulation (FIG. 2B, green). Additionally, in the presence of biapigenin the fast decrease in TMRM signal was attenuated (FIG. 2B, middle). Kaempferol (10 μM) was ineffective in protecting from delaying calcium deregulation. Of all the three tested compounds, kaempferol significantly increased calcium influx in cultured hippocampal neurons due to exposure to kainate plus NMDA (not shown); while quercetin and biapigenin had no effect in either basal intracellular calcium concentrations, or in the response elicited by exposure to KCl (30 mM) or 100 μM kainate plus 100 μM NMDA (data not shown). MK-801 (10 μM) significantly inhibited loss of calcium homeostasis (75% reduction, comparatively to 100 μM kainate plus 100 μM NMDA; data not shown). Additionally, atractyloside (40 μM) and bongkrekic acid (16 μM), two inhibitors of the mitochondrial ANT (Dahout-Gonzalez *et al.*, 2005), decreased by 63% and 35% the total number of cells suffering calcium deregulation (data not shown).

Quercetin and Kaempferol Efficiently Inhibit ROS Generation Following Excitotoxic Insults to Cultured Hippocampal Neurons

The evaluation of peroxide production following exposure of cultured hippocampal neurons to

100 μM kainate plus 100 μM NMDA resulted in a clear increase in DCF fluorescence (1.39 fold above control, Table I). Pre-incubation with quercetin and kaempferol (10 μM) significantly inhibited generation of ROS following exposure to the excitotoxins (45% and 39% inhibition, respectively; p <0.01, when compared to kainate plus NMDA); whereas, biapigenin had no significant effect in ROS generation. As a positive control, 35 min incubation with 10 mM H_2O_2 resulted in a similar increase in ROS generation (1.54 fold above control).

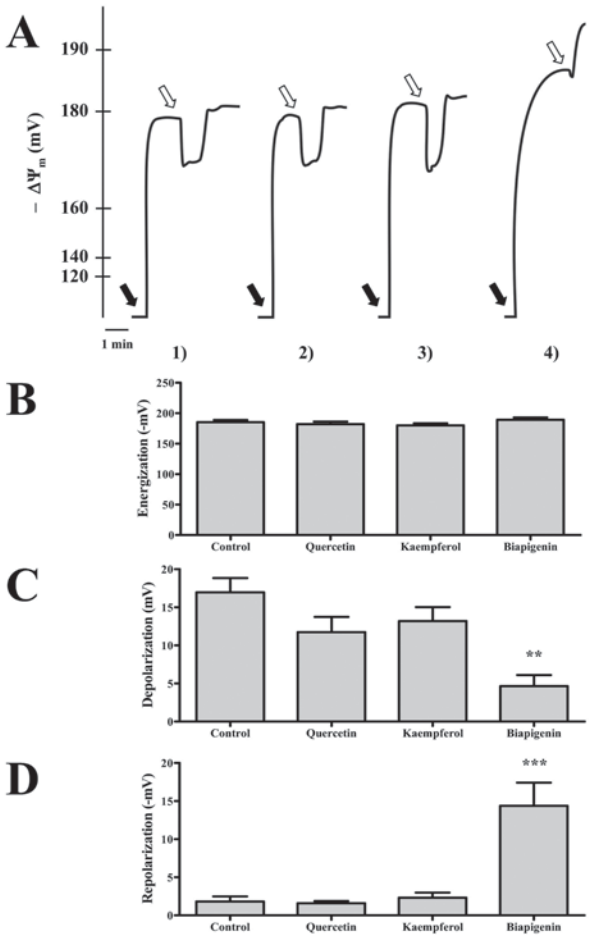

FIGURE 3 Changes in mitochondrial transmembrane potential ($\Delta\psi_m$) upon energization of mitochondria with succinate. **A**) Representative recording for each experimental conditions: 1, control; 2, quercetin (10 μM); 3, kaempferol (10 μM); and 4, biapigenin (10 μM). Black arrows indicate addition of 8 nmol succinate; white arrows indicate addition of 125 μmol ADP. **B**) Mitochondrial transmembrane electric potential upon energization with succinate (control, - 185 ± 4 mV); **C**) after ADP induced depolarization (control, 17 ± 2 mV); and **D**) upon repolarization (control, - 2 ± 1 mV). Values are presented as mean ± SEM (n=3 to 5 independent experiments); **p <0.01, ***p <0.001 (comparatively to the control).

Effect of *H. perforatum* Phenolic Compounds on Mitochondrial Bioenergetics

The results obtained in neuronal cultures and above described contributed to further investigate mitochondrial function as a possible player in the observed neuroprotective effects. For this, we used isolated brain mitochondrial frac-

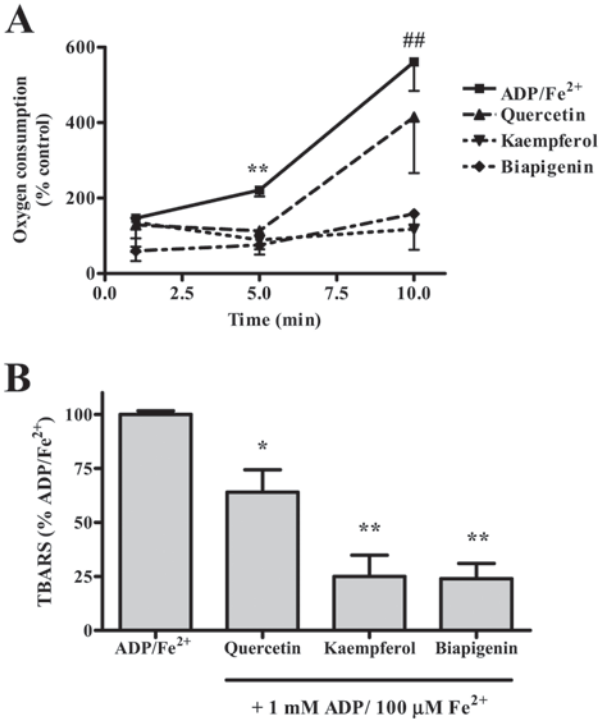

FIGURE 4 Quercetin, kaempferol and biapigenin significantly reduced lipid peroxidation induced by ADP/ iron in isolated brain mitochondria. **A**) Lipid peroxidation induced by 1 mM ADP/ 100 μM $FeSO_4$ (filled square) was significantly reduced in the presence of quercetin (filled triangle, point up), kaempferol (filled triangle, point down) and biapigenin (filled diamond) (10 μM for all three compounds) **p <0.01 for all compounds, ##p <0.01 for kaempferol and biapigenin (when compared with ADP/iron). **B**) Levels of TBARS, measured at the end of the experiment, were significantly reduced in the presence of the three compounds. Values are shown as mean ± SEM (n=3 to 4 independent experiments); *p <0.05, **p <0.01 (comparatively to ADP / iron).

tions in order to directly investigate the effect of quercetin, kaempferol and biapigenin on mitochondrial bioenergetics.

Accordingly to figure 3 and Table II quercetin and kaempferol (10 μM) did not significantly affect mitochondrial TPP uptake and respiration. Incubation of brain mitochondria for 3 minutes with biapigenin (10 μM) significantly affected mitochondrial depolarization and repolarization (FIG. 3C-3D). By itself biapigenin did not significantly affect the TPP electrode response (n=4, data not shown) and respiratory capacity, but decreased state 3 respiration (30% reduction). It is notable that state 4 respiration was also significantly increased (43% increase), which further contributed to the reduction of the respiratory control ratio (RCR - parameter that reflects coupling between substrate oxidation and ADP phosphorylation, 47% decrease comparatively to the control). Uncoupled respiration was also inhibited, which suggests inhibition of the respiratory chain. Moreover, the ADP/O ratio (reflecting phosphorylative efficiency) was also reduced by incubation with biapigenin (approximately 30%). The phosphorylative efficiency was also evaluated through the time required by energized mitochondria to phosphorylate ADP (lag phase), and was significantly reduced by biapigenin (75% inhibition as compared with control, p <0.001 - data not shown).

Mitochondrial Lipid Peroxidation is Reduced in the Presence of Phenolic Compounds from *H. perforatum*

Lipid peroxidation was indirectly evaluated after exposure of isolated brain mitochondria to the oxidant pair ADP/iron. The three compounds were able to significantly reduce oxygen consumption and TBARS production by ADP/iron (FIG. 4A and 4B, respectively). Mitochondria alone (without addition of the pro-oxidants or the protective compounds) did not consume oxygen; and the same was observed for isolated mitochondria incubated with 10 μM of the compounds (data not shown).

The antioxidant properties of the compounds present in *H. perforatum* were also evaluated by inducing lipid peroxidation in energized mitochondria. Mitochondria were energized with succinate and then exposed to ADP/iron. Loss of $\Delta\psi_m$ followed, mainly as a result of loss of membrane integrity, and consequent dissipation of the protonic gradient (FIG. 5). Butylhydroxytoluene (BHT), a well-known inhibitor of lipid peroxidation was used as a control, and completely inhibited loss of $\Delta\psi_m$, further supporting the involvement of lipid peroxidation in this process. Quercetin and kaempferol were very efficient in preventing the loss of $\Delta\psi_m$. Interestingly, energization of mitochondria pre-incubated with biapigenin resulted in a transient hyperpolarization just before peroxidation started.

TABLE I Formation of intracellular peroxides in hippocampal neurons exposed to 100 μM kainate plus 100 μM NMDA (values reported as fold increase relatively to controls). Quercetin, kaempferol or biapigenin (10 μM) were pre-incubated for 15 min. H_2O_2 (10 mM) was used as a control. ** p <0.01 (when compared to kainate plus NMDA).

Kainate + NMDA	Quercetin	Kaempferol	Biapigenin	H_2O_2
1.39 ± 0.11	0.77 ± 0.15**	0.85 ± 0.13**	1.37 ± 0.13	1.54 ± 0.32

TABLE II Mitochondrial respiration assessed after incubation with 10 μM compounds. Results are shown as mean ± SEM (percentages comparatively to control).

	State 3	State 4	RCR	ADP/O	Uncoupled respiration[E]
Control	100.00 ± 5.87 [A]	100.00 ± 4.14 [B]	100.00 ± 6.48 [C]	100.00 ± 7.56 [D]	100.00 ± 1.26 [F]
Quercetin	88.95 ± 8.40	109.30 ± 6.85	82.51 ± 6.59	94.27 ± 14.58	77.83 ± 3.46 *
Kaempferol	93.07 ± 8.10	102.7 ± 3.81	90.79 ± 8.64	105.80 ± 7.59	77.54 ± 7.19 *
Biapigenin	74.44 ± 3.84	142.5 ± 19.23*	53.35 ± 4.34 ***	72.05 ± 11.83	69.64 ± 0.70 **

*p <0.05, **p <0.01, ***p <0.001 (comparatively to control).
Absolute values: [A] – 109.2 ± 12.2 and [B] – 55.0 ± 9.6 natoms O/mg prot/min, [C] – 2.2 ± 0.1, [D] – 1.7 ± 0.1, [E] – FCCP induced uncoupled respiration (maximal value), [F] – 154.3 ± 2.0 natoms O/mg prot/min.

Effects of *H. perforatum* Phenolic Compounds on Mitochondrial Calcium Accumulation

Mitochondrial calcium accumulation was evaluated in energized mitochondria by the use of a low affinity calcium-sensitive probe, Calcium Green-5N in the assay medium. Mitochondria were energized with calcium present in the assay medium, and calcium accumulation was followed by a decrease in fluorescence intensity (reflecting decreased calcium concentration in the medium and accumulation in mitochondria). Representative traces are shown in figure 6A. Most interestingly, in the presence of cyclosporine A, we observed that biapigenin significantly reduced calcium accumulation (FIG. 6B). No significant changes were observed after incubation with quercetin and kaempferol. On the other hand, incubation with cyclosporin A alone significantly increased mitochondrial calcium accumulation (FIG. 6B).

The efficiency of mitochondria to accumulate calcium as a response to an increase in cytoplasmatic calcium concentration, (*e.g.*, during excitotoxicity events) was evaluated by adding pulses of 10 μM calcium to energized mitochondria in suspension. Figure 7 shows the representative traces of calcium accumulation in control or after incubation with biapigenin (10 μM) revealing that in the presence of biapigenin mitochondria has a lower calcium uptake capacity.

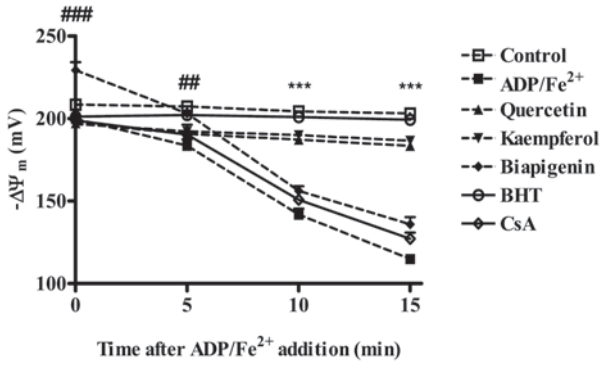

FIGURE 5 Changes in mitochondrial transmembrane electric potential ($\Delta\psi_m$) in mitochondria energized with succinate (empty square), induced by exposure to ADP/iron. Addition of 1 mM ADP/100 μM FeSO4 (filled square) induced dissipation of $\Delta\psi_m$. Loss of $\Delta\psi_m$ was significantly delayed in the presence of the compounds (10 μM for all three compounds). Quercetin (filled triangle, point up) and kaempferol (filled triangle, point down) efficiently prevent the drop in $\Delta\psi_m$ induced by ADP/iron, similarly to the effect observed for BHT (O) (30 μM). Interestingly, biapigenin (filled diamond) caused a significant hyperpolarization of brain mitochondria and delayed the ADP/iron-induced decay in $\Delta\psi_m$, and exhibited a similar profile to cyclosporin A (empty diamond). Values are presented as mean ± SEM (n=4 independent experiments). $^{\#\#}p$ <0.01 for biapigenin, $^{\#\#\#}p$ <0.001 for biapigenin, ***p <0.001 (comparatively to ADP / iron).

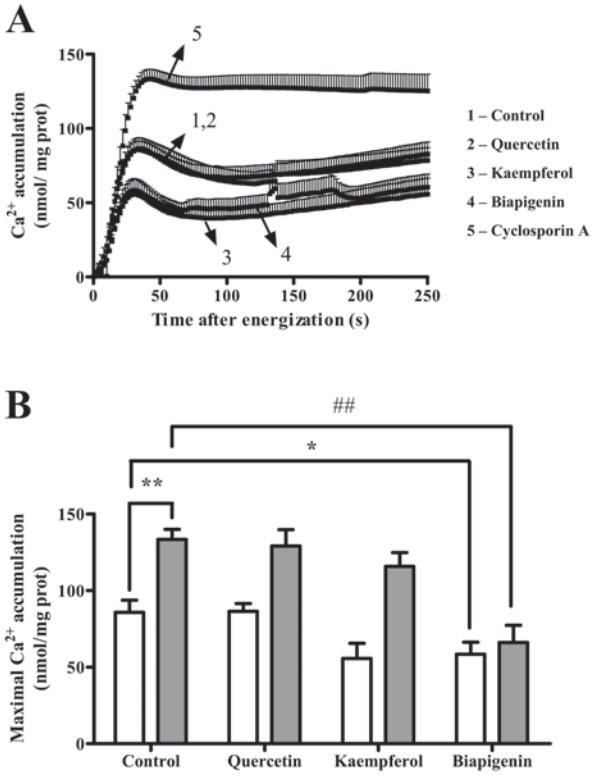

FIGURE 6 Calcium accumulation by brain mitochondria evaluated by following Calcium Green-5N fluorescence. **A**) Figure shows representative recordings of mitochondrial calcium accumulation in control and after incubation with quercetin, kaempferol, biapigenin (10 μM for the three compounds) and cyclosporin A (0.6 μM). Mitochondria were energized, in the presence of calcium (20 μM $CaCl_2$), by addition of 4 mM succinate. Calcium accumulation was monitored by changes in fluorescence intensity following energization. **B**) Biapigenin significantly inhibits calcium uptake and also significantly inhibits cyclosporin-mediated maximal mitochondrial calcium uptake. **Empty bars** - without cyclosporin A (0.6 μM); **full bars** - with cyclosporin A. Values are presented as mean ± SEM (n=3 to 5 independent experiments). *p <0.05 (comparatively to control); $^{\#\#}p$ <0.01 (comparatively to cyclosporin A).

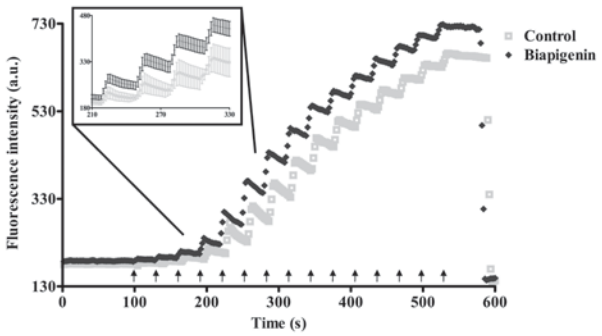

FIGURE 7 Energized mitochondria exposed to pulses of calcium. Representative recordings of calcium accumulation in control (empty square) and after incubation with biapigenin (filled diamond) (10 μM). Pulses of 5 nmoles Ca^{2+}/mg protein were applied (arrows) to energized mitochondria in suspension. At the end of the experiment, EGTA was added. Mitochondrial calcium loading capacity was higher for the control (lower recording), comparatively to calcium accumulation after pre-incubation with biapigenin for 3 min (upper recording). Loss of fluorescence reflects mitochondrial calcium loading capacity. Differences between the two conditions are shown for the time period where mitochondria exhibited higher calcium loading capacity (insert graph), as shown by loss of fluorescence intensity after each calcium pulse. Values are shown as mean ± SEM (*n*=3 to 4 independent experiments).

DISCUSSION

Neuronal death due to excitotoxicity is associated with massive calcium influx, loss of ion homeostasis and mitochondrial dysfunction, usually preceding cell death (Stout *et al.*, 1998). Mitochondria are important organelles for calcium homeostasis, especially under elevated cytoplasmatic calcium concentration resulting from stress conditions (Kristian and Siesjo, 1998; Nicholls, 2002; Weber, 2004; Isaev *et al.*, 2005; Saris and Carafoli, 2005). Calcium entering through glutamate receptors is primarily extruded by the Na^{+}/Ca^{2+} exchanger - NCX (Bano *et al.*, 2005), but it can also be accumulated by mitochondria present in the vicinity of membrane receptors (Peng and Greenamyre, 1998; Weber, 2004), possibly playing a major role in the cellular defense to excessive rise in cytoplasmatic calcium concentration. However, following calcium-induced calpain activation, NCX can be cleaved by the proteolytic activity of calpains (Bano *et al.*, 2005). The intracellular calcium concentration rises above a critical threshold, mitochondria lose the ability to maintain calcium homeostasis and mitochondrial dysfunction occurs. Mitochondrial dysfunction involves the induction of the permeability transition, which can cause mitochondrial swelling and rupture of the outer mitochondrial membrane, releasing pro-apoptotic factors able to trigger processes of cell death (Vander Heiden and Thompson, 1999; Bouchier-Hayes *et al.*, 2005).

The phenols quercetin, kaempferol and biapigenin, present in *H. perforatum* extracts, were able to significantly protect cultured hippocampal neurons against an excitotoxic insult with kainate plus NMDA. Pre-incubation with MK-801, an NMDA-receptor antagonist, prevented excitotoxicity in cultured hippocampal neurons.

Single cell calcium imaging studies supplied evidence for a protective effect of biapigenin against excitotoxic injury, since this compound partially/significantly protected cultured neurons from delayed calcium deregulation, but it did not significantly affect peak calcium rise or initial TMRM decrease in fluorescence, presumably due to plasma membrane depolarization. Furthermore, calcium deregulation was strongly correlated with a late onset fast decline in mitochondrial transmembrane electric potential, suggesting a close association between the two events. Treatment with biapigenin afforded a significant protection to cells suffering early calcium deregulation and decreasing the total number of cells losing calcium homeostasis; with a parallel protection from mitochondrial depolarization. These data might suggest that mitochondria can be a target useful for neuroprotective strategies involving protection from calcium deregulation afforded by biapigenin.

Evidences from the literature are not conclusive about the role of oxidative stress as a cause or a consequence of mitochondrial dysfunction in excitotoxicity. It is uncertain whether mitochondrial dysfunction resulting from excessive calcium uptake is responsible for increased generation of ROS, or if calcium-dependent ROS generation is associated with toxic mechanisms responsible for mitochondrial failure (Lafon-Cazal *et al.*, 1993; Kiedrowski and Costa, 1995; Reynolds and Hastings, 1995; Weber, 2004). In agreement with our data, quercetin and kaempferol can be consid-

ered good antioxidants (Jovanovic and Simic, 2000; Cotelle, 2001; Ishige *et al.*, 2001; Rice-Evans, 2001), and previous studies have reported that both compounds are effective inhibitors of lipid peroxidation (Filipe *et al.*, 2001; Schroeter *et al.*, 2001; Ozgova *et al.*, 2003; Peng and Kuo, 2003).

In accordance, we also observed that quercetin and kaempferol are strong inhibitors of peroxide generation and peroxidation of mitochondrial membranes. Studies with liver mitochondria suggest that quercetin is a potent inhibitor of mitochondrial respiration and a potent inhibitor of mitochondrial membrane permeability transition (Santos *et al.*, 1998; Dorta *et al.*, 2005). However, it must be stressed that the inhibitory effects reported in the studies with liver mitochondria were observed for concentrations significantly higher than those used in our study. Quercetin and kaempferol were reported to have an inhibitory effect in the mitochondrial ATPase/ATP synthase activity. Again, these effects were observed using higher concentrations of both compounds (Zheng and Ramirez, 2000). Montero and colleagues (2004) reported a significant effect of kaempferol in mitochondrial calcium uptake, involving activation of the mitochondrial calcium uniporter in HeLa cells; such effect was less pronounced when quercetin was tested.

Our data from single cell calcium imaging studies indicate that kaempferol increased calcium rise induced by kainate plus NMDA exposure and aggravates delayed calcium deregulation, whereas quercetin was without major significant effect (data not shown). On the other side, both compounds were significantly neuroprotective against excitotoxicity-induced neuronal degeneration, suggesting that the main mechanism underlying neuroprotection might be related to their antioxidant properties. A recent report from Angeloni and colleagues (2007) suggested that inhibition of caspase-3 activation could be a possible mechanism of cellular protection exerted by quercetin against oxidative stress in H9c2 cell lines. Moreover, previous studies reported kaempferol to inhibit caspase-3 activation and to prevent ROS generation (Schroeter *et al.*, 2001; Wang *et al.*, 2001; Samhan-Arias *et al.*, 2004). Therefore, it seems likely that, although kaempferol might increase calcium influx/recruitment from intracellular stores, it might also protect neuronal function by reducing oxidative stress and onset of apoptosis.

The antioxidant properties of quercetin and kaempferol can contribute to maintain the structural and functional properties of mitochondrial respiratory chain, essential for mitochondrial function and generation of the proton-motive force. Moreover, we also observed that the antioxidant properties of these compounds were efficient against peroxide production and lipid peroxidation caused by oxidative stress induced in non-energized but also in energized mitochondria. The hyperpolarizing effect observed after incubation of mitochondria with biapigenin can contribute to the lower antioxidant efficiency of this compound in energized mitochondria, comparatively to the strong protection observed after induction of oxidative stress in non-energized mitochondria. However, and interestingly, the protection afforded by biapigenin against transmembrane potential decay was very similar to that observed in the case of cyclosporin A, a well known inhibitor of the opening of the permeability transition pore (Halestrap, 2006); while on the other hand, the profiles exhibited by quercetin and kaempferol were much closer to that of BHT, a known potent inhibitor of lipid peroxidation, highlighting possible differences in neuroprotective mechanisms triggered by quercetin/kaempferol or by biapigenin.

As mentioned above, excitotoxicity is closely associated with increased intracellular calcium concentration and compromised mitochondrial function, whereas decreased mitochondria calcium accumulation seems to mediate neuroprotection under excitotoxic insults (Stout *et al.*, 1998; Dubinsky *et al.*, 2004). One could hypothesize that the protective mechanisms of biapigenin can be somewhat related with modulation of mitochondrial calcium accumulation, possibly decreasing calcium retention. Nevertheless, biapigenin also appears to interfere with the mitochondrial phosphorylative mechanisms, significantly reducing the ADP-induced depolarization, and the time required to phosphorylate added ADP. Additionally, the ADP/O ratio, which reflects the efficiency of phosphorylation, was also significantly reduced after incubation with biapigenin. Further work is being carried out in our laboratory to explore this subject.

Mitochondrial calcium accumulation depends on both the rate of calcium uptake and release.

Enhanced calcium release can be a consequence of the induction of the mitochondrial permeability transition (MPT), triggered by excessive calcium accumulation and oxidative stress. The sustained opening of MPT is prevented in fully polarized mitochondria or in the presence of antioxidants (Vieira *et al.*, 2000; Brookes *et al.*, 2004). Biapigenin reduced mitochondrial calcium loading capacity when evaluated directly with a fluorescent probe (Calcium Green-5N), which may explain why it was able to increase the maximal membrane potential attained in the presence of calcium. The data obtained from mitochondrial calcium accumulation points to an interesting effect of biapigenin, which might also help explain the observed neuroprotection in neuronal cells. Despite the need for further work, the results suggest that biapigenin could contribute to the maintenance of mitochondrial function through a mechanism that can involve modulation of mitochondrial calcium accumulation under stressful conditions of calcium overload. Another possible explanation for the phenomenon could be that biapigenin can increase the rate of calcium extrusion. Accordingly, we observed a reduction in calcium accumulation by mitochondria incubated with both biapigenin and cyclosporin-A.

We are aware of the difficulty in comparing results obtained in intact cells with results obtained with isolated mitochondria. Whether or not the effects in mitochondrial function by biapigenin contribute to neuroprotection is a debatable matter. Nevertheless, compounds that interact with mitochondrial calcium systems (*e.g.*, the calcium uniporter or the mitochondrial permeability transition) can have antagonism or synergism over drugs that modulate cytosolic calcium, and by these processes influence cell viability. Our data suggests that the interaction of biapigenin with mitochondrial calcium homeostasis can be associated with the observed neuroprotection in cultured neurons.

The present study indicates that phenolic compounds present in *H. perforatum* extracts are neuroprotective against excitotoxicity in cultured hippocampal neurons, involving antioxidant properties. Moreover, in the specific case of biapigenin we show that it prevents onset of calcium deregulation and mitochondrial dysfunction, possibly, by processes related with mitochondrial calcium homeostasis.

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