Quercetin Attenuates Oxygen–Glucose Deprivation- and Excitotoxin-Induced Neurotoxicity in Primary Cortical Cell Cultures

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The possible role of quercetin, a naturally occurring plant flavonoid, in protecting against oxygen–glucose deprivation (OGD)-, excitotoxins-, and free radical-induced neuronal injury in mouse cortical cell cultures was investigated. Pre- and co-treatment with quercetin (100 μM) inhibited 50 min OGD-, 20 μM *N*-methyl-D-aspartate (NMDA)-, and 50 μM kainate-induced neurotoxicity by 36, 22, and 61%, respectively. Quercetin significantly ameliorated free radical-induced neuronal injury caused by buthionine sulfoximine, sodium nitroprusside, $ZnCl_2$, and $FeCl_2$. These results suggest that quercetin may contribute a neuroprotective action against ischemic neural injury, partially *via* antioxidant actions.

Key words quercetin; oxygen–glucose deprivation; *N*-methyl-D-aspartate; kainate; free radical; cortical culture

The abrupt elevation of reactive oxygen species and lipid peroxidation during brain damage plays a key role in various neurodegenerative processes, such as cerebral ischemia, trauma, Parkinson's and Alzheimer's diseases.[1,2] Quercetin, a natural flavonoid, is well known as a constituent of *Ginkgo biloba* and *Hypericum perforatum* (St. John's wort), and occurs in other fruits and vegetables.[3,4] In recent studies, quercetin decreased neuronal injury in cultured hippocampal cells by inhibiting production of nitric oxide[5] and metal ion-induced peroxidation of lipids.[6] Quercetin inhibits the release of PGE_2, expression of cyclooxygenase-2, and inducible nitric oxide synthase,[7] although quercetin may have some unfavorable effects under ischemic conditions, such as inhibiting ischemic tolerance.[8] In this study, we examined whether quercetin protects neuronal cells in primary mouse cortical cultures against neurotoxicity induced by oxygen–glucose-deprivation (OGD) or excitotoxins [*N*-methyl-D-aspartate (NMDA) or kainate]. We also determined the inhibitory action of quercetin on various free radical injuries.

MATERIALS AND METHODS

Neuronal and Glial Cultures Mixed cortical cell cultures, containing both glia and neurons, were prepared from ICR mice at 15—16 d of gestation, generally as previously described.[9] Briefly, dissociated neocortical cells (2.5×10^5 cells/well) were plated onto primaria-coated 24-well plates (Falcon) containing a glial bed in plating medium consisting of Eagle's minimal essential medium (MEM; Earle's salts, supplied glutamine-free) supplemented with 20 mM glucose, 2 mM L-glutamine, 5% fetal bovine serum, and 5% horse serum. Cytosine arabinoside (10 μM) was added 5 d after plating to halt the growth of non-neuronal cells. Cultures were maintained at 37 °C in a humidified CO_2 incubator and used for experiments between days 12 and 14 *in vitro*. Glial cultures were prepared from postnatal (1—3 d) mice and plated at 0.75 hemispheres/24-well plate in plating medium supplemented with 10% horse serum, 10% fetal bovine serum, and 10 ng/ml epidermal growth factor. After 2 weeks *in vitro*, cytosine arabinoside was added to the cultures, which were fed weekly with the same medium with 10% horse serum used for mixed cultures. We added 10 μM glycine (final concentration) to all the media used in this study. Pretreatment of cultured cells with quercetin for 20—24 h before initiating all neuronal damages was executed, and added it again during and after the exposure of OGD, or during other neuronal injuries.

Oxygen–Glucose Deprivation (OGD) Simultaneous oxygen and glucose deprivation was brought about by abruptly switching the culture medium to glucose-free, deoxygenated Earle's balanced salt solution (BSS_0; dilution$>$ 1 : 1000) in an anaerobic chamber as previously described.[10] OGD was terminated by switching the culture medium to oxygenated MEM containing 5.5 mM glucose and 2 mM glutamine, and returning the cultures to a normoxic CO_2 incubator.

Measurement of Neuronal Injury and Immunocytochemistry Neuronal damage was quantitatively estimated by measuring lactate dehydrogenase (LDH) released from damaged cells into the extracellular medium, as previously described.[11] For morphological confirmation, neurons were stained with neuron-specific enolase antibody (Dako, Denmark). Briefly, mixed cortical cultures were fixed for overnight at 4 °C in a freshly prepared solution of 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), washed with PBS three times, and permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. After washing PBS, cultures were incubated in 10% normal goat serum (NGS) in PBS for 30 min at room temperature. After blocking, cultures were labeled with mouse anti-neuron specific enolase (1 : 400) overnight at 4 °C. After three washes, appropriate biotinylated secondary antibody (Goat anti-mouse IgG) was diluted 1 : 200 and added to the cultures for 1 h at room temperature. All antibodies were diluted in PBS containing 2% NGS. The reaction product was visualized using 3,3-diaminobenzidine tetrahydrochloride (Vector, Burlingame, CA, U.S.A.). Images were photographed with an Olympus IX-70 microscope.

Statistical Analysis Data are expressed as the mean± standard error of the mean (S.E.M.) and analyzed for statisti-

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cal significance by one-way analysis of variance (ANOVA) using a post-hoc Student–Neuman–Keuls test for multiple comparisons.

RESULTS AND DISCUSSION

Exposure of mixed cortical cell cultures to 20 μM NMDA for 20—24 h caused LDH release to increase by approximately 70—80%. When 100 μM quercetin was present both before and during NMDA exposure, neuronal damage was reduced by 22% ($p<0.01$) (Fig. 1). Quercetin (100 μM) also inhibited 50 μM kainate-induced neurotoxicity by 61% ($p<0.01$) (Fig. 1). When cultures were subjected to OGD by switching from oxygenated MEM to BSS_0 for 50 min, neuronal swelling was observed soon after the switch. At approximately 20—24 h after OGD terminated, approximately 60—70% of the neuronal cells were damaged. When 10 μM MK-801 or 100 μM quercetin was re-added to the cultures during and after the OGD period, neuronal injury was reduced by 84 and 36%, respectively ($p<0.01$) (Fig. 1). Morphologically, OGD-induced losses of neuronal cell bodies and neuritis were apparent 20—24 h after cessation of OGD (Fig. 2B). However, cell bodies and neurites in the quercetin-treated group were healthier than those of controls (Fig. 2D).

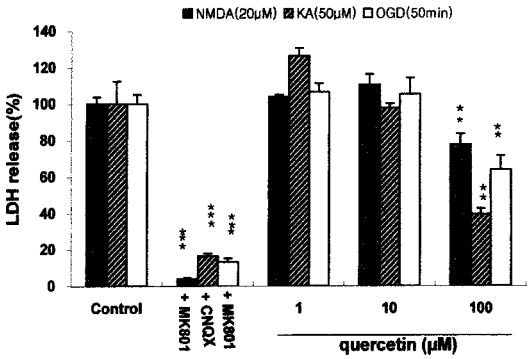

Fig. 1. Pre- and Co-treatment with Quercetin (100 μM) Attenuates *N*-Methyl-D-aspartate (NMDA)-, Kainate (KA)- and Oxygen–Glucose Deprivation (OGD)-Induced Neurotoxicity

Bars represent LDH release (mean±S.E.M., $n=4$) in sister cultures after 20—24 h of exposure to 20 μM NMDA or 50 μM KA, or after 50 min of OGD (controls), or with the addition of 10 μM MK-801 or 50 μM CNQX, or with the addition of quercetin at the indicated concentrations. The differences were evaluated with one-way ANOVA and the post-hoc Student–Neuman–Keuls test for multiple comparisons (**$p<0.01$, ***$p<0.001$ *vs.* controls).

Exposure of neuronal cultures to 1 mM buthionine sulfoximine (BSO), an inhibitor of endogenous glutathione synthesis or 40 μM $FeCl_2$ induced 70—80% neuronal injury as evaluated by elevation of LDH release into the bathing medium after 20—24 h. Pre- and co-treatment of cultured neurons with 1—10 μM quercetin for 20—24 h did not have a neuroprotective effect on BSO- and $FeCl_2$-induced neurotoxicity. However, 30—100 μM concentration of quercetin significantly reduced the neurotoxicity induced by BSO and $FeCl_2$ by 27—34% and 75—92%, respectively (Table 1). When the cultures were exposed to 50 μM sodium nitroprusside (SNP), which directly produces nitric oxide, submaximal neuronal damage was observed after 20—24 h. Quercetin at concentrations ranging from 10 to 100 μM almost blocked SNP-induced neuronal injury by 97—93% (Table 1). However, all tested concentrations of quercetin (1—100 μM) significantly inhibited the neurotoxicity induced by 30 μM $ZnCl_2$ (Table 1). In this study, our results did not suggest direct evidence

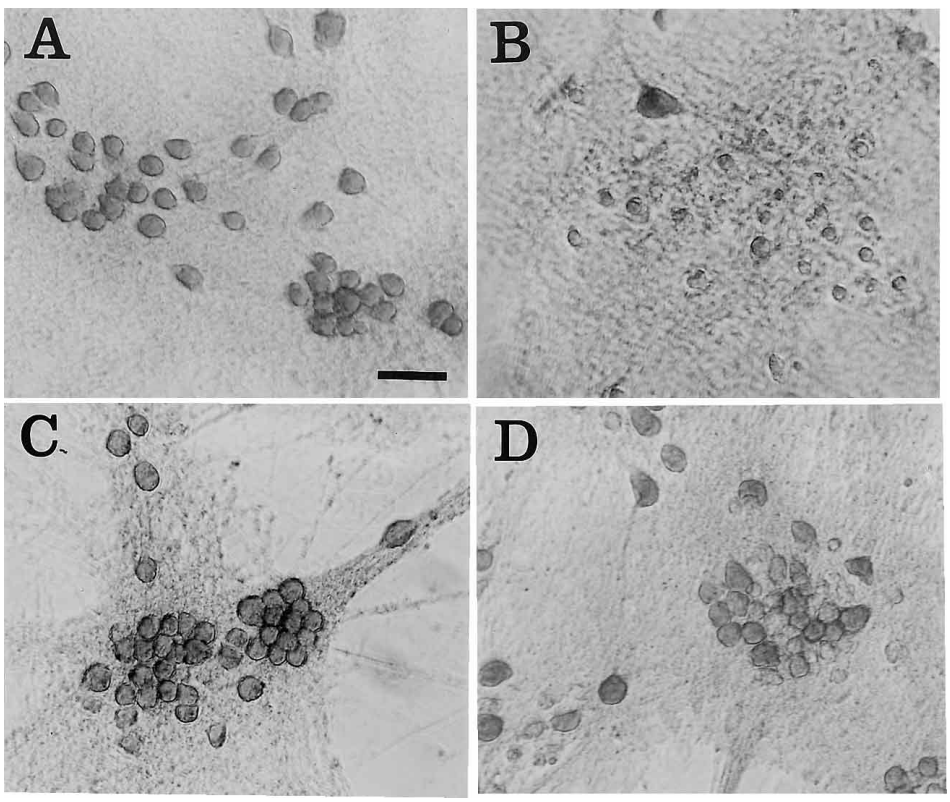

Fig. 2. Morphological Evidence of Neuroprotection with Quercetin in Oxygen–Glucose Deprivation (OGD)-Induced Neurotoxicity

Phase-contrast photomicrographs of sister cultures 20—24 h after a 50 min exposure to OGD alone (B), or in the presence of 10 μM MK-801 (C), or 100 μM quercetin (D), or sham wash (A). Neurons were stained with neuron-specific enolase antibody (Dako, Denmark), Scale bar, 50 μm.

Table 1. Antioxidant Effects of Quercetin on Free Radical-Induced Neuronal Injury

	LDH Released			
	BSO (1 mM)	$FeCl_2$ (40 μM)	SNP (50 μM)	$ZnCl_2$ (30 μM)
Control	100±17.7	100±5.0	100±7.0	100±8.6
Quercetin				
1 μM	106.5±15.9	124±10.2	101.6±13.7	41.6±19.5*
10 μM	97.9±5.8	109±9.9	2.7±3.4**	6.3±0.9**
30 μM	73.4±3.8*	25.4±14.2**	N.D.	N.D.
100 μM	66.3±15.9*	8.2±1.6**	6.8±4.6**	32.9±5.7*

Cortical cultures were exposed to 1 mM buthionine sulfoximine (BSO), 40 μM $FeCl_2$, 50 μM sodium nitroprusside (SNP), or 30 μM $ZnCl_2$ in the presence or absence (control) of 1±100 μM quercetin. LDH levels in the medium were measured 24 h (5 h in SNP study) after exposure and are given as the mean±S.E.M. (n=4). The differences were evaluated with one-way ANOVA and the post-hoc Student–Neuman–Keuls test for multiple comparisons (*p<0.01, **p<0.001 *vs.* controls). N.D.; not determined.

on relationship between the inhibitory effects of quercetin against OGD- or excitotoxins-induced neurotoxicity and antioxidant actions. But, oxidative stress is well known to participate in the cascade process of neuronal injury during the exposure of OGD or NMDA in cultured neurons.[12,13] We already have identified that some antioxidant compounds, such as phenidone, dual inhibitor of cyclooxygenase and lipoxygenase or eugenol, one of the plant phenylpropanoid derivatives, exhibited a neuroprotective effect against NMDA and OGD neurotoxicity.[9,14] In this study, the antiexcitotoxic and antioxidant properties of quercetin were shown to protect cortical cells from OGD-induced neurotoxicity.[15] Reactive oxygen species and lipid peroxidation are generated in the early phase of Zn^{2+}-induced neurotoxicity,[16] and combined Zn^{2+} accumulation and increased free radical generation have been observed in brains after transient global ischemia.[17] Nitric oxide has been known to have a dual effect, neuronal protection or destruction depending on the difference of neuropathological situation.[18] However, in case of OGD-induced neuronal injury, the elevation of inducible nitric oxide synthase (iNOS) expression can exacerbate the neuronal damage.[19] Therefore, chelation of Zn^{2+} and scavenge of nitric oxide (·NO) by quercetin may be a beneficial factor in ameliorating neuronal injury subsequent to cerebral ischemia.

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