Quercetin-Induced PC12 Cell Death Accompanied by Caspase-Mediated DNA Fragmentation

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Flavonoids have been reported to be potent antioxidants and beneficial in oxidative stress related diseases. Quercetin, a major flavonoid in food, deserves much attention because of its antioxidative activity. However, the actions of flavonoids including quercetin are complex and paradoxical. Quercetin caused apoptosis and/or cell death in various cells including cancer cells and normal cells. In this study, we investigated the effects of quercetin with or without hydrogen peroxide (H2O2) on cell death of PC12 cells, a neuronal cell line. We showed that quercetin at 10–30 μM alone caused cell death accompanied by caspase-mediated DNA fragmentation in undifferentiated PC12 cells. Quercetin did not inhibit and rather enhanced 0.1 mM H2O2-induced cell death. The toxic effect of quercetin was not inhibited by antioxidants such as N-acetylcysteine and GSH, although H2O2-induced cell death was inhibited by the antioxidants. Quercetin-induced cell death was reduced by 2 h treatment with nerve growth factor and serum. In addition, quercetin caused cell death in differentiated PC12 cells that were cultured with nerve growth factor for 6 d. Genistein, a soy isoflavone that has the pro-apoptotic activity, also caused cell death with DNA fragmentation. Further evaluation of the potential of dietary flavonoids as neuroprotective reagents is needed.

Key words quercetin; apoptosis; caspase; DNA fragmentation; reactive oxygen species; PC12 cell

Flavonoids, a large group of polyphenolic compounds found in fruits and vegetables, have attracted much attention because of their broad pharmacological, particularly antioxidative, activities. Flavonoids may exert beneficial effects on a number of disease states including cancer, cardiovascular disease, inflammation and neurodegenerative disorders. The pathology of neurodegenerative disorders including Alzheimer’s disease and aging is associated with oxidative stress caused by reactive oxygen species and reactive nitrogen species. If oxidative stress is suppressed by compounds in foods, such as flavonoids, this may act as a preventive measure to reduce damage and/or dysfunction of cells. Quercetin (3,3′,4′,5,7-pentahydroxyflavone), which possesses a wide spectrum of pharmacological and biological properties, is found in many kinds of food. The daily Western diet contains on average about 25 mg of various flavonoids, of which quercetin is a major ingredient (16 mg/d). Quercetin can scavenge free radicals directly and inhibit the oxidation of various molecules resulting in the activation of antioxidant defense pathways in vivo and in vitro. Quercetin and its analogs have been established as useful in protecting various neuronal cells against oxidative stress.

However, the actions of flavonoids are complex and paradoxical in some cases. Several studies have shown that quercetin is a pro-oxidant and cytotoxic in leukemic cells. Flavonoids including quercetin caused apoptosis in carcinoma/cancer cells and showed cytotoxicity in normal cells including human umbilical vein endothelial cells. The neuronal density of the hippocampus in gerbils subjected to infusion of quercetin to the ventricle was significantly lower than that in control. Thus, the effects of quercetin on cell death and/or apoptosis in cells including neuronal cells have not been well confirmed.

The PC12 cell line, a rat adrenal medullary pheochromocytoma, is a useful model for studying the survival of neuronal cells. Native PC12 cells divide and resemble precursors of adrenal chromaffin cells and sympathetic neurons when grown in the presence of serum, and gradually attain the phenotypic properties of sympathetic neurons on the addition of nerve growth factor (NGF). Previously, we reported that the treatment of native and undifferentiated PC12 cells with an inhibitor of thioredoxin reductase and dopaminergic neurotoxins such as 1-methyl-4-phenylpyridinium ion (MPP+) caused apoptosis via a caspase-mediated pathway. Quercetin has been shown to inhibit cell death and/or apoptosis induced by various stimuli including oxidative stress and MPP+ in differentiated PC12 cells. In undifferentiated PC12 cells, however, antioxidants including quercetin did not have a protective effect against cell death induced by oxidative stress including MPP+. The effect of quercetin on cell survival and death in undifferentiated PC12 cells has not been well established. In the present study, we investigated whether quercetin causes cell death in undifferentiated PC12 cells or not. The effect of genistein, a soy isoflavone that has the pro-apoptotic activity, was also investigated.

MATERIALS AND METHODS

Materials The cytotoxicity detection kit used for the measurement of lactate dehydrogenase (LDH) activity was from Roche (Mannheim, Germany). Quercetin, rutin and rutinose were obtained from Dr. K. Masaki (Toyo Suisan, Ltd., Tokyo, Japan). Genistein, 1-chloro-2,4-dinitrobenzene (DNCB), N-acetylcysteine and GSH-methyl ester were purchased from Sigma (St. Louis, MO, U.S.A.). z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) was purchased...
from Biomol (Plymouth Meeting, PA, U.S.A.). NGF (2.5S) and H$_2$O$_2$ were obtained from Wako (Osaka, Japan).

**Culture of PC12 Cells** PC12 cells were grown in collagen-coated dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (Thermo-Trace, Melbourne, Australia), as described previously. For experiments, sub-confluent PC12 cells 24 h after the final change of medium were cultured with DMEM containing 0.2% serum in the presence of vehicle or quercetin for 24 h. In some experiments, PC12 cells were treated every 3 d with NGF (50 ng/ml) in DMEM containing 0.5% serum, and used after 6 d for experiment. To verify the effect of the caspase inhibitor, the cells were precultured for 1 h with medium containing 50 $\mu$M zVAD-fmk. To verify the effects of NGF and serum, the cells were cultured for 2 h with medium containing 50 ng/ml NGF or 5% serum before the treatment with quercetin. The concentrations of N-acetylcysteine and GSH-ethyl ester were selected based on previous studies. Quercetin, genistein and zVAD-fmk were dissolved in dimethyl sulfoxide and diluted with the medium. The final concentration of dimethyl sulfoxide in the medium was 0.5%. Other reagents including NGF were dissolved in water and diluted with the medium. The vehicles with and without dimethyl sulfoxide did not show toxicity at 24 or 48 h.

**LDH Leakage Assay** Cell death was estimated by the LDH leakage method as described previously. LDH leakage (%) was defined as the ratio of LDH activity in the culture medium to total activity ($\% = (extracellular\ activity)/(extracellular\ activity\ and\ remaining\ cellular\ activity) \times 100$).

**Measurement of DNA Fragmentation** DNA fragmentation was measured with an Apoptosis Screening Kit (Wako) using TdT-mediated dUTP nick end labeling (TUNEL). The procedures were in accordance to the manufacturer’s directions. Since the absolute values of OD$_{450}$ in the control cells changed depending on the individual experiment (0.40—0.75, $n = 3$), the data were presented as fold-stimulation of the control value obtained with vehicle.

**Statistical Analysis** Values are presented as mean± S.E.M. from three or more independent experiments. The statistical significance for differences between two groups was assessed using a two-tailed Student’s t-test. Multiple comparisons against a single control group were made by a one-way analysis of variance followed by the Bonferroni test. $p<0.05$ was considered to be statistically significant.

**RESULTS**

**Quercetin-Induced Stimulation of PC12 Cell Death** In PC12 cells, serum-deprivation alone caused marked cell toxicity. In the present study, PC12 cells were treated with reagents in the DMEM containing 0.2% serum. First, we investigated whether quercetin has a protective effect against H$_2$O$_2$-induced cell death in undifferentiated PC12 cells (Table 1). Treatment with 16 $\mu$M quercetin for 1 h, which alone had no effect on LDH leakage, did not inhibit the leakage of LDH induced by 0.1 mM H$_2$O$_2$ 24 h after the H$_2$O$_2$ was added. Treatment with 33 $\mu$M quercetin significantly caused LDH leakage by itself, and did not inhibit the H$_2$O$_2$-induced cell death. Treatment with quercetin at 1, 4 or 8 $\mu$M for 1 h and simultaneous addition of quercetin at various concentrations with 0.1 mM H$_2$O$_2$ did not have a protective effect on the H$_2$O$_2$-induced cell death; the values of LDH leakage were almost same as those without quercetin treatment (ca. 30%). Rutin, a glycoside of quercetin with rutinose, at 50 $\mu$M is reported to prevent MPP$^+$-induced cell death in differentiated PC12 cells. However, rutin by itself (16 and 32 $\mu$M, 100 and 200 $\mu$g/ml, respectively) only slightly increased LDH leakage (the values were 13—15%), and did not protect the cells against 0.1 mM H$_2$O$_2$ (the values were 40—45%). Rutinose at 153 $\mu$M (50 $\mu$g/ml) and the lower concentrations (15, 30 $\mu$M) had no toxic effects by itself and did not show a protective effect against 0.1 mM H$_2$O$_2$. Previously, we reported that treatment with an inhibitor of thioredoxin reductase DNCB at 10—20 $\mu$M for 4 h caused cell death accompanied by the activation of caspases in undifferentiated PC12 cells. Treatment with quercetin at 1 to 20 $\mu$M for 1 h did not inhibit the DNCB-induced LDH leakage over a period of 4 h in representative experiments. The values obtained with 10 $\mu$M DNCB were ca. 20% in the control and the quercetin (10 $\mu$M)-treated cells, while those obtained without DNCB were ca. 5% in both cells, in a typical experiment. These results show that quercetin does not have a protective effect against cell death in undifferentiated PC12 cells. Under our conditions, quercetin alone stimulated LDH leakage and the effects of quercetin and H$_2$O$_2$ were additive in differentiated PC12 cells treated with NFG for 6 d (Table 1). Undifferentiated PC12 cells were used in the following experiments.

**Quercetin-Induced LDH Leakage and DNA Fragmentation and Their Inhibition by Caspase Inhibitor and NGF** The LDH leakage induced by quercetin was concentration-dependent, and treatment with 50 $\mu$M zVAD-fmk for 1 h almost completely inhibited the quercetin-induced LDH leakage 24 h after the addition (Fig. 1, Panel A). Treatment with 20 $\mu$M zVAD-fmk significantly inhibited the leakage of LDH induced by 66 $\mu$M quercetin; the value in the zVAD-fmk-treated cells was 24.5±3.7% ($n = 3$). Quercetin at greater concentrations than 165 $\mu$M caused a further LDH leakage (more than 80%), and the inhibitory effect of 50 $\mu$M zVAD-fmk was not seen clearly (about 60—70%) because of this high level of LDH leakage. Thus, the ED$_{50}$ value of quercetin for zVAD-fmk-sensitive LDH leakage (30±6 $\mu$M, 18—45 $\mu$M, $n = 4$) was calculated using 165 $\mu$M quercetin as a maximal value. The addition of 66 $\mu$M quercetin significantly caused DNA fragmentation as assayed by the TUNEL method 24 h after the addition (Fig. 1, Panel B). Addition of 165 $\mu$M genistein, which is a soy isoflavone reported to cause

<table>
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<tbody>
<tr>
<td>None</td>
<td>10.7±0.6</td>
<td>32.9±1.9*</td>
</tr>
<tr>
<td>Quercetin (16 $\mu$M, 5 $\mu$g/ml)</td>
<td>10.9±0.5</td>
<td>39.9±0.8*</td>
</tr>
<tr>
<td>(33 $\mu$M, 10 $\mu$g/ml)</td>
<td>28.3±2.0*</td>
<td>42.3±4.0*</td>
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Table 1. Effect of Quercetin on H$_2$O$_2$-Induced Cell Death in PC12 Cells

Undifferentiated and differentiated PC12 cells were cultured with DMEM containing 0.2% serum in the presence of the indicated concentrations of quercetin for 1 h and then with vehicle or 0.1 mM H$_2$O$_2$ for an additional 24 h. Data are means±S.E.M. for three independent experiments done in triplicate. *$p<0.05$, significantly different from the values in the un-treated control cells.
apoptosis in several cancer cell types\textsuperscript{21,22}) induced DNA fragmentation in PC12 cells. Addition of 0.1 mM H$_2$O$_2$ also caused DNA fragmentation. Treatment with 50 $\mu$M zVAD-fmk significantly inhibited quercetin-, genistein- and H$_2$O$_2$-induced DNA fragmentation.

Undifferentiated PC12 cells were cultured with 50 ng/ml NGF for 2 h and then further cultured with vehicle, 33 and 66 $\mu$M quercetin, and 165 $\mu$M genistein (Fig. 2, Panel A). NGF treatment significantly inhibited the LDH leakage induced by quercetin and genistein. Treatment with 50 ng/ml NGF for 24 h inhibited the response by half (data not shown). PC12 cells were cultured with fresh DMEM with and without 5% serum for 2 h and then further cultured with vehicle, and 66 and 165 $\mu$M quercetin for 24 h. The treatment with serum significantly inhibited the LDH leakage induced by quercetin (Panel B). The inhibitory effect of serum on quercetin-induced cell death was partial not complete. Treatment with 5% serum inhibited the leakage of LDH induced by 165 $\mu$M quercetin by half in a typical experiment; the values are means±S.E.M. for three independent experiments done in triplicate. *$p<0.05$, significantly different from the value without zVAD-fmk.

leakage induced by 165 $\mu$M genistein was partly (about 30—50%) inhibited by serum treatment.

Quercetin-Induced Cell Death Independent of Reactive Oxygen Species Production of reactive oxygen species and/or redox status are known to play an important role in the intracellular signaling pathway of apoptosis. Treatment with quercetin decreased the GSH concentration in cells.\textsuperscript{23,24)} Next, the effects of antioxidants N-acetylcysteine and GSH-ethyl ester were examined. First, PC12 cells were cultured with 25 mM N-acetylcysteine or 2 mM GSH-ethyl ester for 24 h in DMEM containing 0.2% serum, and then cells were cultured for 24 h with 66 $\mu$M quercetin without the antioxidants for the LDH leakage assay. The treatments did not inhibit quercetin-induced LDH leakage (Table 2), although the treatments almost completely inhibited the leakage of LDH.
induced by 0.1 mM H₂O₂. The treatments alone had no toxic effect. The treatments did not inhibit 33 μM quercetin-induced LDH leakage; the values were about 30% in vehicle-, N-acetylcysteine- and GSH-ethyl ester-treated cells. LDH leakage induced by 165 μM genistein was not inhibited in the antioxidant-treated cells (data not shown).

DISCUSSION

Quercetin-Induced Cell Toxicity in Undifferentiated PC12 Cell
We showed that quercetin (aglycone) caused cell death measured as LDH leakage, but rutin (glycone, quercetin plus rutinose) and rutinose did not have toxic effects in undifferentiated PC12 cells. Our results show that the quercetin-induced cell death is probably apoptosis accompanied by caspase-mediated DNA fragmentation, and treatment with neurotrophic factors such as NGF and serum inhibited the toxic effect of quercetin. The ED₅₀ value of quercetin required for the death of PC12 cells was about 30 μM, which is similar to the value reported for the toxic effect of quercetin in other cells.25,26 Quercetin did not protect against H₂O₂-induced cell death in undifferentiated PC12 cells at any of the concentrations tested. Under our conditions, rutin did not show quercetin-like toxic effect in PC12 cells, as previously reported in human promyeloleukemic HL-60 cells25 and endothelial cells.19 Some reports showed that rutin could not show quercetin-like effect on undifferentiated and differentiated PC12 cells.29,29 However, both rutin and quercetin showed similar effect,20,26 and quercetin-3-α-galactoside showed protective effects against cytotoxicity induced by oxidative stress in PC12 cells.31 The process for deglycosylation of rutin may be dependent on subclone of PC12 cells and/or culture conditions.

Quercetin is known to conjugate with various materials including serum albumin in human plasma, with no significant amounts of free quercetin present.32 Thus, deprivation of free quercetin may be caused by serum treatment. However, serum treatment inhibited cell death induced by low and high concentrations in a similar degree (Fig. 2, Panel B). Also, the toxicity of quercetin was half that in PC12 cells treated with serum for 2 h and then cultured with quercetin in the absence of serum. Like NGF, factors in serum may activate putative signaling pathways leading to inhibition of apoptosis, and not the detoxification of quercetin through, for example, the formation of a complex. Quercetin undergoes various chemical modifications such as methylation, glucuronidation, and/or sulfation. After the incubation of 10 μM quercetin with 3T3 fibroblasts for 4 h, isorhamnetin (3,4’,5,7-tetrahydroxy-3’-methoxyflavone) and glucuronide/sulfate conjugates of isorhamnetin had accumulated in the medium, but the level of isorhamnetin including the metabolites was quite low in cellular fractions including cell membranes.33 By contrast, substantial amounts (ca. 6% of the initial content) of quercetin and its glucuronide/sulfate conjugates were present in cellular fraction. Quercetin 3-O-β-glucuronide was less effective than quercetin in suppressing the formation of oxygen species and was not taken up into differentiated PC12 cells.34 Thus, quercetin not its metabolites appears to be effective in our study, although the cellular responses (death and survival) were different.

Possible Mechanism of Quercetin-Induced Cell Toxicity
Flavonoids including quercetin have been described as possessing pro-oxidative activity, and mutagenic and/or anti-cancer activity,7,27 in addition to anti-oxidative activity. Treatment with N-acetylcysteine and GSH-methyl ester, which have been shown to act as a precursor for the biosynthesis of GSH and as radical scavengers, inhibited the generation of reactive oxygen species and apoptosis in neuronal cells including PC12 cells induced by various stimuli such as reactive oxygen species.17,18 However, they did not suppress the quercetin-induced cell death in PC12 cells. Thus, the formation of reactive oxygen species does not appear to be involved in the toxicity of quercetin in undifferentiated PC12 cells.

Heat shock proteins such as Hsp27 and Hsp70 act as chaperons in cells, and the enhanced expression of the proteins reduced the toxicity induced by MPP⁺ in PC12 cells.35 Since treatment with quercetin inhibited the expression of heat shock proteins in PC12 cells,31 quercetin may induce cell toxicity via changes in expression of the proteins. Accumulating evidence suggests that flavonoids including quercetin and genistein have the potential to bind to the ATP-binding sites of various proteins including ATPases and protein kinases such as a family of mitogen-activated protein kinases.2 The reasons for this discrepancy are not clear at present. It should be determined whether quercetin has an anti-oxidative effect in intracellular levels under our conditions. Treatment with quercetin may stimulate the putative signals for cell death described above, which may offset the protective effects including anti-oxidative activity induced by quercetin. It is reported that treatment with quercetin at concentrations greater than 10 μM for 6 h caused cell death in primary cortical neurons.25 Quercetin showed a protective effect against MPP⁺ toxicity in differentiated, but not in undifferentiated, PC12 cells.15 These reports may indicate that the response to quercetin differs depending on the degree of cell differentiation. In our conditions, however, quercetin caused cell death of differentiated PC12 cells. Green tea polyphenol exerted different effects in altering the oxidative stress, GSH metabolism and reactive oxygen species production in different subcellular compartments in PC12 cells.36 Flavonoids including quercetin were capable of regulating the cytochrome P450 enzymes.36 The apoptotic response to quercetin may be de-
dependent on intracellular conditions of cells such as redox state and mitochondria function, in addition to the level of apoptosis-related proteins as stated above.

It is apparent that the cell death and/or apoptosis of PC12 cells caused by quercetin can confuse the interpretation of results drawn from studies that use quercetin as an antioxidative reagent in the brain and neurons. Nowadays, flavonoids are being taken as dietary supplements for good health. Quercetin is not present as an aglycon and occurs only in the conjugated forms such as quercetin-3-O-glucuronide in plasma. The concentrations in plasma in human reached ca. 1.5 μM/l after 28 d of supplementation with a high dose of quercetin (>1 g/d). Bioavailability of quercetin differs among food sources depending on the type of glycosides they contain, and the plasma concentrations reached 7.6 μM after an intake of 100 mg quercetin equivalents in onions.

Very high interindividual variability of the levels of quercetin after an intake of 100 mg quercetin equivalents in onions was drawn from studies that use quercetin as an antioxidants agent. Among other factors, quercetin can confuse the interpretation of results from studies that use quercetin as an antioxidative reagent in the brain and neurons. Nowadays, flavonoids are being taken as dietary supplements for good health. Quercetin is not present as an aglycon and occurs only in the conjugated forms such as quercetin-3-O-glucuronide in plasma. The concentrations in plasma in human reached ca. 1.5 μM/l after 28 d of supplementation with a high dose of quercetin (>1 g/d). Bioavailability of quercetin differs among food sources depending on the type of glycosides they contain, and the plasma concentrations reached 7.6 μM after an intake of 100 mg quercetin equivalents in onions.

Very high interindividual variability of the levels of quercetin was observed possibly because of polymorphism for intestinal enzymes or transporters, and the practical concentration of quercetin in neuronal tissues has not been elucidated. In view of its toxicity, an appropriate intake of quercetin should be borne in mind.

Acknowledgments

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