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Effect of fraxetin and myricetin on rotenone-induced cytotoxicity in SH-SY5Y cells: comparison with *N*-acetylcysteine

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Abstract

The purpose of this study was to investigate the potential neuroprotective effects of myricetin (flavonoid) and fraxetin (coumarin) on rotenone-induced apoptosis in SH-SY5Y cells, and the possible signal pathway involved in a neuronal cell model of Parkinson's disease. These two compounds were compared to *N*-acetylcysteine. The viability of cells was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and cytotoxicity was assayed by lactate dehydrogenase (LDH) released into the culture medium. Parameters related to apoptosis, such as caspase-3 activity, the cleavage of poly(ADP-ribose) polymerase and the levels of reactive oxygen species were also determined. Rotenone caused a time- and dose-dependent decrease in cell viability and the degree of LDH release was proportionally to the effects on cell viability. Cells were pretreated with fraxetin, myricetin and *N*-acetylcysteine at different concentrations for 30 min before exposure to rotenone. Cytotoxicity of rotenone (5 μM) for 16 h was significantly diminished as well as the release of LDH into the medium, by the effect of fraxetin, myricetin and *N*-acetylcysteine, with fraxetin (100 μM) and *N*-acetylcysteine (100 μM) being more effective than myricetin (50 μM). Rotenone-induced apoptosis in SH-SY5Y cells was detected by an increase in caspase-3 activity and in the cleavage of poly(ADP-ribose) polymerase. After exposing these cells to rotenone, a significant increase in reactive oxygen species preceded apoptotic events. Fraxetin (100 μM) and *N*-acetylcysteine (100 μM) not only reduced rotenone-induced reactive oxygen species formation, but also attenuated caspase-3 activity and poly(ADP-ribose) polymerase cleavage at 16 h against rotenone-induced apoptosis. The effect of fraxetin in both experiments was similar to that of *N*-acetylcysteine. These results demonstrated the protective action of fraxetin and suggest that it can reduce apoptosis, possibly by decreasing free radical generation in SH-SY5Y cells. Myricetin at 100 μM was without any preventive effect.

Keywords: Flavonoid; Rotenone; Oxidative stress; SH-SY5Y cell; Parkinson's disease

1. Introduction

Oxidative stress and mitochondria play an important role in the control of most physiological processes, cell injury and programmed cell death (Kehrer and Smith, 1994). A number of reports point to the presence of ongoing oxidative stress and inflammatory processes occurring selectively in the substantia nigra pars compacta of parkinsonian brains (Jenner and Olanow, 2003; Levites et al., 2002). Decreases in the mitochondrial respiratory chain complex activities as well as mitochondria-derived oxidative stress have been implicated in a number of diseases such as those related to neurodegeneration and chemical-induced cell injury/death. Rotenone, a plant-derived pesticide, is a specific inhibitor of mitochondrial complex I and has been shown to produce effects in rats which closely resemble Parkinson's disease (Greenamyre et al., 2001; Wang et al., 2002). It also been shown that rotenone is able to induce apoptosis via enhancing the generation of mitochondrial reactive oxygen species production (Li et al., 2002; Sipos et al., 2003).

Antioxidants, free radical scavengers and other such drugs have the potential for therapeutic development used to prevent Parkinson's disease. Flavonoids and coumarins comprised a group of phenolic compounds widely distributed in natural plants, and they have recently attracted much attention because of their pharmacological activities. They have multiple biological activities, including anticarcino-

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genic, anti-inflammatory, antibacterial, antiviral, antithrombotic effects, etc. (Hladovec, 1977). Many flavonoids, for example myricetin and many coumarins such as fraxetin, showed scavenging activity against reactive oxygen species and inhibits lipid peroxidation in rat brain (Martin-Aragon et al., 1997; Proteggente et al., 2002; Rice-Evans, 2001; Rice-Evans and Miller, 1996; Rice-Evans et al., 1995; Ng et al., 2000). Although the physiological benefits of flavonoids have been largely attributed to their antioxidant properties in plasma, flavonoids may also protect cells from various insults. Some previous studies report that *Ginkgo biloba*, possibly through the antioxidant properties of its flavonoids, was able to protect hippocampal cells against toxic effects induced by Abeta peptides (Bastianetto and Quirion, 2002) and possesses protective effect on the Parkinson's disease models in vitro (Yang et al., 2001). Recently, some investigations have been done on the protective effect of flavonoids on the neuron; the results showed that flavonoids with high effectiveness with respect to scavenging free radicals could protect neuronal cells from oxidative stress (Gao et al., 1999, 2001; Sher et al., 1992; Ni et al., 1996). Esculetin (6,7-dihydroxycoumarin) and other coumarins, using rodent (tracheal epithelial or liver) cells and human cells (neonatal foreskin fibroblasts, bronchial epithelial cells, or human leukemic cells [HL-60]), inhibit the carcinogenesis process and are identified as candidates for development as chemopreventive agents (Kawaii et al., 2000; Sharma et al., 1994). However, the effects of these compounds, myricetin and fraxetin, on oxidative stress of cultured cells, especially on neuronal cells, are relatively unknown. Considering the antioxidant properties of myricetin and fraxetin and phytonutrients and their possible therapeutic efficacies, the purpose of the present study was to investigate the effects of pretreatment with myricetin and fraxetin on rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells in order to find a possible therapeutic application of these natural compounds to degenerative diseases. We used *N*-acetylcysteine, a known antioxidant agent, to verify the effect of these compounds.

2. Materials and methods

2.1. Reagents

Reagents were from Sigma (St. Louis, MO, USA), Boehringer (Mannheim, Germany) and Merck (Darmstadt, Germany). RPMI 1640 and fetal bovine was purchased from Gibco. The fluorescent probes 2′7′-dichlorohydrofluorescein diacetate (DCFH-DA) was purchased from Sigma and caspase-3 II fluorigenic substrate (Ac-DEVD-AMC) were from Calbiochem (Darmstadt, Germany). Antibody for poly(ADP-ribose) polymerase (Pharmingen/Transduction Laboratories, San Diego, CA). Human neuroblastoma SH-SY5Y cells came from Dr. Guillermo Repetto from the National Institute of Toxicology (Sevilla, Spain).

2.2. Cell culture and drug treatment

Human neuroblastoma SH-SY5Y cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 50 μg/ml gentamycin in a water-jacketed incubator at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were plated at a density of 10^5 cells/60-mm dish and were cultured for 48 h prior to treatments. Prior to all treatments, cells were rinsed twice with serum-free medium containing 50 μg/ml gentamycin and were incubated in serum-free media at least 1 h before adding the treatment. In a pilot investigation, cells were treated with rotenone at concentrations ranging from 5 to 50 μM for various intervals and then examined for cell viability. Concentrations of 5 μM rotenone (dissolved in dimethyl sulfoxide [DMSO]; final medium concentration: 0.05% DMSO) or vehicle as the control were used in an extensive study of the markers of cell death after 16 h exposure. Myricetin dissolved in DMSO (the final concentration was 50 μM and the DMSO content should never exceed 0.2%), 100 μM fraxetin dissolved in absolute ethanol (the final medium concentration: 0.2% ethanol) and *N*-acetylcysteine (100 μM) dissolved in the RPMI medium were added 0.5 h prior to rotenone treatment.

2.3. Analysis of cell viability

Cell viability could be quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Kitamura et al., 1998). SH-SY5Y cells were seeded in 96-well plates at a density of 12,500 cells/well. The cultures were grown for 24 h, the medium was changed to that containing various concentrations of rotenone and the pretreatment. After 16 h incubation, 100 μl of MTT reagent (0.2 mg/ml MTT in PBS containing 10 mM HEPES) was added to each well. Following an additional 1 h incubation at 37 °C, 100 μl of DMSO was added to dissolve the formazan crystals, and the absorbance was then measured at 595 nm using a Digiscan Microplate Reader (Assys Hitech, Kornenburg, Austria). Wells without cells were used as blanks and were subtracted as background from each sample. Results were expressed as a percentage of control.

2.4. Lactate dehydrogenase release

The cells were placed in 24-well plates at a density of $1.5–2 \times 10^4$ cells/well and were allowed to attach for 24 h before treatment. One row contained the medium only for background subtraction. Myricetin, fraxetin and *N*-acetylcysteine were added 30 min before rotenone for a subsequent 16 h. The supernatant was collected for lactate dehydrogenase (LDH) measurement in the cell-free medium. The cell pellet and the cells remaining on the multiwell were lysed in 0.5 ml of lysis buffer (0.5% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.0). The release of

intracellular LDH to the extracellular medium was measured by determining this enzyme activity and was expressed as a percentage of total cellular activity (Suuronen et al., 2000). The absorbance was measured at 340 nm using a model Digiscan Microplater Reader.

2.5. Measurement of caspase-3 activity

Fluorometric assays of caspase-3 activity were conducted as described previously (Bijur et al., 2000) in 96-well clear-bottom plates, and all measurements were carried out in triplicate wells. To each well, 200 μl of assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM dithiothreitol) was added. The peptide substrate for caspase-3 (Ac-DEVD-AMC) was added to each well to a final concentration of 25 ng/μl. Cells were lysed with 100 μl of lysis buffer without sodium orthovanadate and okadaic acid. The lysates were collected in microcentrifuge tubes, sonicated and centrifuged at 14,000 × *g* for 10 min at 4 °C. Protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) method. Cell lysates (20 μg protein) were added to start the reaction. Fluorescence was measured on a fluorescence plate reader at 360 nm excitation and 460 nm emission. Caspase activity was calculated as [(mean AMC fluorescence from triplicate wells) − (background fluorescence)]/micrograms of protein.

2.6. Immunoblot analysis

Cell lysates were mixed with Laemmli sample buffer (2% sodium dodecyl sulphate (SDS)) and placed in a boiling water bath for 5 min. Proteins were resolved in a 7.5% SDS–poyacrylamide gel, transferred to nitrocellulose and incubated with antibodies for poly(ADP-ribose) polymerase. Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse, followed by detection by enhanced chemiluminescence (King et al., 2001).

2.7. Reactive oxygen species determination

Viable cells (10^4/well) were plated into 24-well plates 24 h before the experiments. On the day of the experiments, after removing the medium, the cells in the plates were incubated with 5 μM DCFH-DA in serum-free medium in 5% CO_2/95% air at 37 °C for 30 min. After DCFH-DA was removed, the cells were washed and incubated with RPMI without phenol red containing different concentrations of the following compounds, rotenone, myricetin, fraxetin and *N*-acetylcysteine, and fluorescence of the cells from each well was measured and recorded in the multiwell fluorescence plate reader. The fluorescence intensity (relative fluorescence units) was measured at 485 nm excitation and 530 nm emission in a model 7620 Microplate Fluorometer. Data points were taken every 15 min for 2 h (Wang et al., 1999).

2.8. Statistical analysis of the data

Experimental values are the mean ± S.D. values of the number of experiments. One-way analysis of variance (ANOVA) followed by a Newman Keuls' multiple comparison test was used to compare control and treated groups with P values <0.05 being considered statistically significant.

3. Results

3.1. Parameter of cell viability and cytotoxicity

The loss of cell viability in culture is generally measured by the reduction of MTT activity and the release of LDH into the media. The survival of SH-SY5Y cells exposed to oxidative stress is shown in Fig. 1. Treatment of SH-SY5Y cells with rotenone caused cell death in a concentration- and time-dependent manner (Fig. 1A and B). In treatments for 16 h, observe that a dose of 5 μM of rotenone was enough to reduce the viability by 20% and the MTT activity was sharply decreased above 50 μM. Fig. 1A show also the effects of myricetin, fraxetin and *N*-acetylcysteine, without

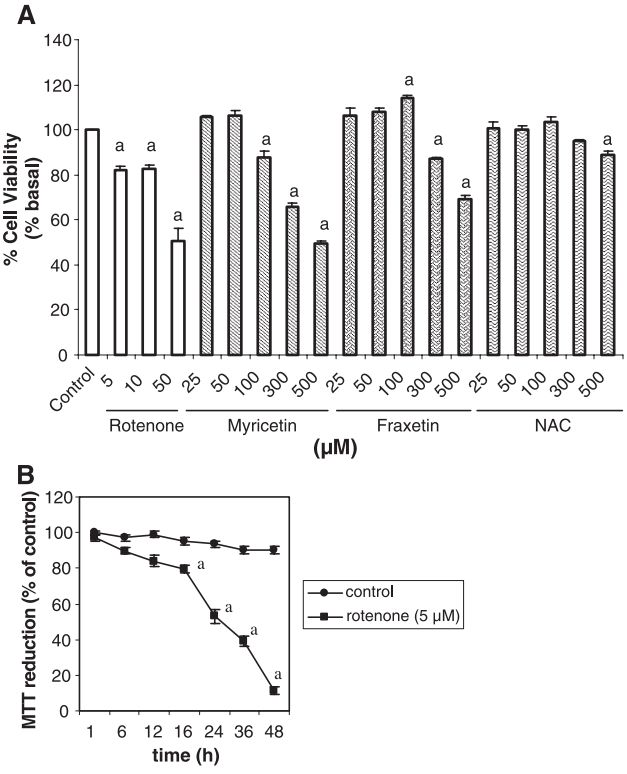

Fig. 1. Treatments toxicity. (A) Effects of different concentrations of the rotenone and myricetin, fraxetin and *N*-acetylcysteine on cell viability in normal SH-SY5Y cells after treatment for up to 16 h. (B) Time course of cell viability from SHSY-5Y cells by treatment with 5 μM rotenone. Cell viability was measured by MTT method. Date are expressed as percent of values in untreated control cultures and are means ± S.D. of four experiments. [a]$P<0.05$ compared to control.

rotenone, on cell viability of SH-SY5Y cells after incubating with different concentrations using the MTT assay. Fraxetin showed light cytotoxicity at higher concentrations (500 μM). *N*-acetylcysteine treatment itself did not cause any cytotoxic effects up to the highest concentration used (500 μM) and showed similar viability as untreated control cells. It should be noted that at high concentrations (100–500 μM), myricetin decreased cell viability, but when myricetin concentrations decreased to lower than 50 μM, no cytotoxicity was shown. Among the tested compounds, myricetin showed the highest toxicity to the cells.

The fraxetin and *N*-acetylcysteine protects against the neurotoxicity induced by 5, 10 and 50 μM rotenone. The myricetin had minor but no significant effect (data not shown).

Exposure of SHSY-5Y cells to 5 μM rotenone for 8 h or less did not induce the release of LDH from the cells, but a pronounced LDH release occurred following 16 h of treatment (Fig. 2A). Treatment with rotenone resulted in a significant increase in LDH release in a dose-dependent manner (Fig. 2B). Preincubation of SHSY-5Y cells with 50 μM of myricetin or 100 μM of fraxetin for 30 min protected the cells against the toxicity of rotenone (Fig. 2C). *N*-acetylcysteine (100 μM) significantly prevented rotenone-induced cell death.

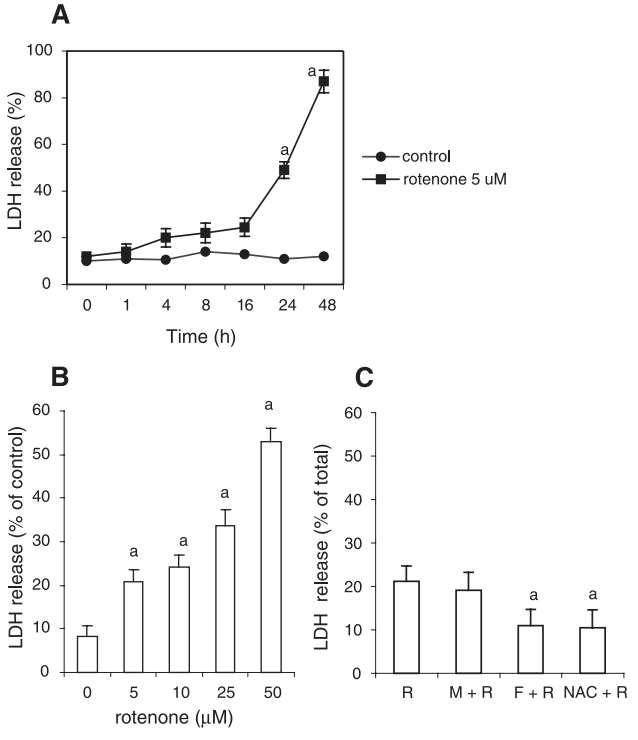

Fig. 2. Cytotoxicity measured by LDH release. (A) Time course of LDH release from SHSY-5Y cells by treatment with rotenone. (B) Treatment with rotenone resulted in significant increase in LDH release in a dose-dependent manner at 16 h. $^{a}P<0.05$ versus control cell. (C) SHSY-5Y cells were pretreated with the compounds 30 min before the addition of rotenone for 16 h. LDH release induced by 5 μM rotenone (R) was inhibited by, 50 μM myricetin + 5 μM rotenone (M + R), 100 μM fraxetin + 5 μM rotenone (F + R), 100 μM *N*-acetylcysteine + 5 μM rotenone (NAC + R). $^{a}P<0.05$ compared to group treated with rotenone only. Experimental values are the mean ± S.D. values of four determinations.

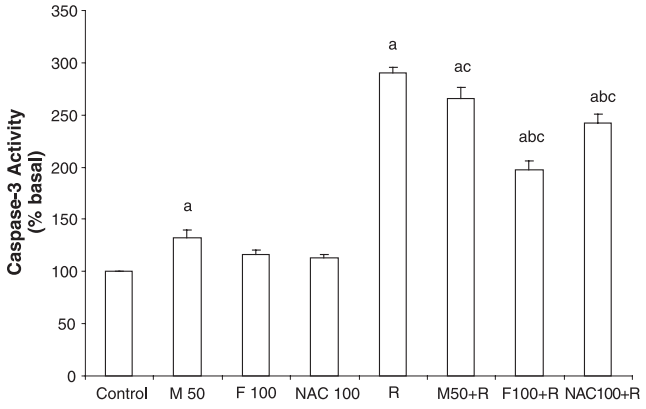

Fig. 3. Caspase-3 activity. Effect of myricetin, fraxetin or *N*-acetylcysteine pretreatment on caspase-3 activity in rotenone-treated SH-SY5Y cells for 16 h. Control, 50 μM myricetin (M), 100 μM fraxetin (F), 100 μM *N*-acetylcysteine (NAC), 5 μM rotenone (R), 50 μM myricetin + 5 μM rotenone (M50 + R), 100 μM fraxetin + 5 μM rotenone (F100 + R), 100 μM *N*-acetylcysteine + 5 μM rotenone (NAC100 + R). $^{a}P<0.05$ compared with control without rotenone treatment. $^{b}P<0.05$ versus group treated with rotenone only. $^{c}P<0.05$ versus group treated with the same antioxidant treatment without rotenone. Data are means ± S.D. of four independent experiments.

3.2. Assay for caspase-3 activity

The level of caspase-3 activity is a good quantitative parameter for neuronal apoptosis (Marks et al., 1998). In this study, we evaluated caspase activity by assessing proteolysis of poly(ADP-ribose) polymerase (the best characterized substrate for caspases) by Western blot, as well as measuring relative levels of activated caspase-3, using the fluorogenic caspase-3 substrate DEVD-AMC. Following 16 h treatment of SHSY-5Y cells with 5 μM rotenone, we detected an increase to almost threefold the control value of caspase-3 activity. SH-SY5Y cells treated with 50 μM myricetin or 100 μM fraxetin for 30 min prior to exposure to rotenone resulted a significant decrease in caspase-3 activity (by 21% and 85%, respectively) compared with rotenone-treated cells (Fig. 3). Moreover, treatment of SH-SY5Y cells with *N*-acetylcysteine (100 μM) 30 min before rotenone exposure reduced caspase-3 activity by 40% with respect to cells treated only with rotenone.

The cleavage of poly(ADP-ribose) polymerase was assessed by Western blot to determine whether this key regulator of apoptosis was involved in rotenone-induced neurotoxicity. Poly(ADP-ribose) polymerase (116 kDa) was cleaved to generate the smaller 85-kDa fragment after 16 h of exposure to 5 μM rotenone. The untreated control sample (without rotenone) did not display poly(ADP-ribose) polymerase cleavage (Fig. 4, lane 1). Pretreatment with 50 μM myricetin showed partial inhibition of PARP cleavage induced by rotenone (Fig. 4, lane 3); however, pretreatment with 100 μM mirycetin induced a dramatic increase in

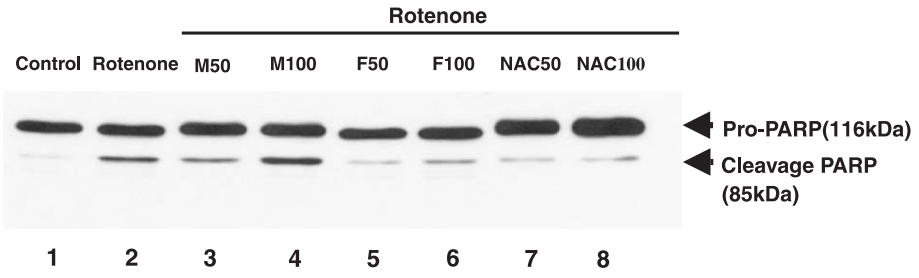

Fig. 4. Pretreatment with 50 or 100 μM myricetin (M50 and M100), 50 or 100 μM fraxetin (F50 and F100) and 50 or 100 μM *N*-acetylcysteine (NAC50 and NAC100) attenuated rotenone-induced poly(ADP-ribose) polymerase cleavage in SHSY-5Y cell as determined by Western blotting. The 116-kDa band is the uncleaved full-length poly(ADP-ribose) polymerase, whereas the 85-kDa band represents the apoptotic cleavage. Lane 1 control (without rotenone); lane 2, 5 μM of rotenone for 16 h; lanes 3–8, pretreatment with myricetin, fraxetin or *N*-acetylcysteine for 30 min prior to 5 μM rotenone for 16 h.

DEVD-specific caspase activity in treated SH-SY5Y cells with rotenone (Fig. 4, lane 4). Fraxetin (50 and 100 μM) or *N*-acetylcysteine (50 and 100 μM) almost completely inhibited the poly(ADP-ribose) polymerase cleavage (Fig. 4, lanes 5–8).

3.3. Effect of fraxetin and myricetin on reactive oxygen species production

The degree of reactive oxygen species accumulation after rotenone exposure was measured to determine the role of reactive oxygen species in rotenone neurotoxicity. The accumulation of oxygen free radicals was determined by fluorescence assay. Fig 5 shows that H_2O_2 production by rotenone (5 μM) was detectable as early as 15 min of exposition and reached the maximal production at 75 min. In our experimental condition, pretreatment with myricetin, fraxetin and *N*-acetylcysteine suppressed the generation of reactive oxygen species and significantly decreased the amount of intracellular peroxide levels from 96.4 ± 5.4 (control) to 152.6 ± 23.7 (rotenone), 119.3 ± 9.5 (myricetin [50 μM] + rotenone), 62.3 ± 3.4 (*N*-acetylcysteine [100 μM] + rotenone) and 43.7 ± 16.3 (fraxetin [100 μM] + rotenone) for 90 min.

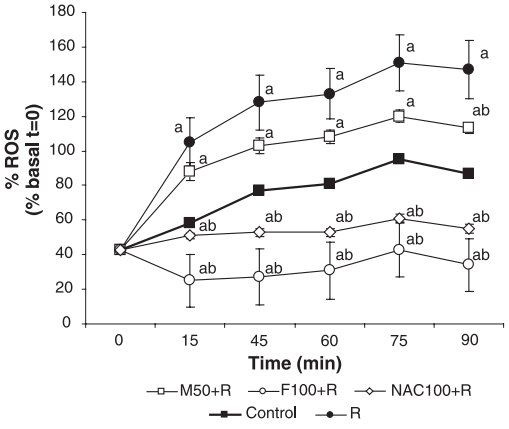

Fig. 5. Time-course changes in peroxides level in SHSY-5Y cells. Pretreatment with 50 μM myricetin, 100 μM fraxetin or 100 μM *N*-acetylcysteine for 30 min prevented oxidative stress induced by exposure to 5 μM of rotenone for 16 h in SHSY-5Y cells. The accumulation of oxygen free radicals was estimated by fluorescence assay. [a]$P < 0.05$ compared with control (■) without rotenone. [b]$P < 0.05$ versus group treated with rotenone only (●). Data are means ± S.D. of four independent experiments.

4. Discussion

Several studies have demonstrated that reactive oxygen species are involved in the apoptotic mechanism of rotenone-mediated neurotoxicity (Wang et al., 2002) and may contribute to the increase in the apoptotic processes found in Parkinson's disease (King et al., 2001). In our study, rotenone induced dose-dependent toxicity in a period of 16 h, as shown in Figs. 1 and 2. Expositions of SH-SY5Y cells cultures with rotenone resulted in an increase in LDH release from the cells and a decrease of the MTT activity. The time course of LDH release showed that the rotenone toxicity is initiated after 16 h of incubation, and marked LDH release was observed after 48 h. Data from this present study show that treatment with rotenone results a significant increase of reactive oxygen species level, this is consistent with previous descriptions of rotenone-induced generation of free radicals. We found that a significant increase in reactive oxygen species formation preceded apoptotic events such as caspase-3 activation and poly(ADP-ribose) polymerase cleavage, events implicated in Parkinson's disease (Wang et al., 2002). Other studies obtained similar results in cortical neuronal cells following exposure with rotenone (Pei et al., 2003).

There is no effective therapy to neurological disorders related to accumulation of free radicals. Among therapeutic interventions that are envisioned to delay the progress of aging-related disease such as Parkinson's disease, nutritional interventions may be viewed as one of the most viable approach. It has also been reported that diets rich in fruits and vegetables are important source of polyphenols. Interestingly, epidemiological studies pointed that a moderate consumption of flavonoids may reduce the incidence of certain neurological disorders including dementia (Rao and Balachandran, 2002; Engelhart et al., 2002; De Rijk et al., 1997).

In the present study, we first demonstrate that fraxetin and *N*-acetylcysteine have significant neuroprotective effects against apoptosis induced by rotenone. Cellular survival was increased significantly in cells treated with these compounds prior to oxidative insult. The protective abilities of myricetin against rotenone-induced cytotoxicity were found to vary widely. Myricetin at high dose shows no protection against rotenone-induced cytotoxicity.

We demonstrated that fraxetin (100 μM) and *N*-acetylcysteine (100 μM) suppressed caspase-3 activation and poly(ADP-ribose) polymerase cleavage. These results are consistent with a variety of studies showing that fraxetin and *N*-acetylcysteine can affect cell death and survival after various types of proapoptotic stimuli (Soto-Otero et al., 2000; Paya et al., 1994).When human endothelial cells were pretreated with fraxetin, as well as with other coumarins, a significant increase in cell survival was observed (Kaneko et al., 2003).

However, activation of caspase-3 associated with poly (ADP-ribose) polymerase cleavage was detected with myricetin (100 μM) in rotenone-treated SH-SY5Y cells, which demonstrates the cytotoxicity of the myricetin at that dose. Other studies, performed on human promyeloleukemic cells, indicate that myricetin induce apoptosis accompanied by activation of caspase-3 (Lee et al., 2002). In spite of coumarins usually more cytotoxic than flavonoids, in this study, we found that fraxetin has better protective action than myricetin which even has proapoptotic effects at high doses (100 μM).

Results from other authors suggest that flavonoids and coumarins can scavenge reactive oxygen species directly (Hoult and Paya, 1996). Previous studies demonstrated that the incubation with myricetin reduced the oxidation of DCFH in resting brain neurons and may be responsible for a part of the beneficial effects of *G. biloba* flavonoids on brain neurons subject to ischemia (Oyama et al., 1992). We have confirmed this observation by using DCFH-DA in our cells. Pretreatment with fraxetin (100 μM), myricetin (50 μM) and *N*-acetylcysteine (100 μM) suppressed the generation of reactive oxygen species, most likely via affecting important enzymatic and nonenzymatic oxidant-scavenging systems. It has previously been reported by our laboratory that fraxetin significantly increased the activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione in mice brain (Martin-Aragon et al., 1997). Moreover, fraxetin prevented oxidative stress by an important increase in antioxidant reserves of GSH, and peroxidative damage is preserved in a *Drosophila melanogaster* experimental model (Fernandez-Puntero et al., 2001). Thus, the net effect of fraxetin treatment on endogenous antioxidant capacity and lipid peroxidation suggests that this compound might provide an important protection against free-radical-mediated events which contribute to degenerative diseases.

One possible underlying mechanism in the effectiveness of fraxetin and myricetin against rotenone neurotoxicity may involve its catechol-like structure, since it is known that catechol-containing polyphenols are potent radical scavengers and chelators of ferric ion (Van Acker et al., 1996; Paya et al., 1993).

The studies on the structure–antioxidant activity relationship of coumarin suggest that orto-catechol moiety in the molecules favours bidentate chelation of iron ions (Paya et al., 1993) and exhibited higher scavenging activity for free radicals than other coumarins without an orto-catechol moiety (Kaneko et al., 2003).

To determine whether suppression of free radical production was sufficient to prevent apoptosis, we employed *N*-acetylcysteine, an agent known as a free radical scavenger, to examine its effects on SH-SY5Y apoptotic cell death induced by rotenone. In the present study, we demonstrated that *N*-acetylcysteine suppressed cell death and caspase-3 activation. These results are consistent with a variety of studies showing that *N*-acetylcysteine protects nerve cells from rotenone neurotoxicity (Li et al., 2002). Experimental studies suggest that efficacy in *N*-acetylcysteine therapy could be valuable in other clinical situations in which GSH deficiency or oxidative stress play a role in disease pathology, e.g., Parkinson's disease (De Rosa et al., 2000).

Therefore, the findings described in this study suggest that the mechanism of rotenone neurotoxicity may involve oxidative stress, and fraxetin may act on reactive oxygen species to inhibit apoptosis by their antioxidant properties.

Further studies of neuroprotective mechanisms of *N*-acetylcysteine, myricetin and fraxetin in detail are necessary before definite conclusions can be drawn.

In summary, these results demonstrate once more the relationship between reactive oxygen species and cell death by apoptosis in a model of in vitro cell damage. Fraxetin and *N*-acetylcysteine markedly protected SH-SY5Y cells from rotenone-induced apoptosis and suppressed rotenone-induced reactive oxygen species generation. In our opinion, the mechanism of rotenone neurotoxicity may involve oxidative stress, and the neuroprotective effects of fraxetin and myricetin may be partly associated with their antioxidant properties.

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