Revised manuscript

Resveratrol protects dopaminergic neurons in midbrain slice culture from multiple insults

Mitsugi Okawara[a], Hiroshi Katsuki[a], Emi Kurimoto[a], Haruki Shibata[a], Toshiaki Kume[a], Akinori Akaike[a,*]

[a]*Department of Pharmacology, Graduate School of Pharmaceutical Sciences,*

Kyoto University, 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

*Address correspondence to: Akinori Akaike, Ph.D.

Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University

46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

Phone: +81-75-753-4550, Fax: +81-75-753-4579

E-mail: aakaike@pharm.kyoto-u.ac.jp

Abstract

Increasing lines of evidence show that resveratrol, a polyphenol compound contained in several dietary products, exhibits cytoprotective actions. Notably, resveratrol activates sirtuin family of NAD-dependent histone deacetylases implicated in regulation of various cellular processes including gene transcription, DNA repair and apoptosis. Here we examined neuroprotective effect of resveratrol on dopaminergic neurons in organotypic midbrain slice culture. Resveratrol and quercetin, another sirtuin-activating polyphenol, prevented the decrease of dopaminergic neurons and the increase of propidium iodide uptake into slices induced by a dopaminergic neurotoxin 1-methyl-4-phenyl pyridinium (MPP^+). Resveratrol also provided concentration-dependent neuroprotective effects against sodium azide, a mitochondrial complex IV inhibitor, and thrombin (EC number 3.4.21.5), a microglia-activating agent. Sirtuin inhibitors such as nicotinamide and sirtinol did not attenuate the protective effect of resveratrol against MPP^+ cytotoxicity. Instead, we found that resveratrol prevented accumulation of reactive oxygen species, depletion of cellular glutathione, and cellular oxidative damage induced by MPP^+, suggesting involvement of antioxidative properties in the neuroprotective action of resveratrol. On the other hand, resveratrol as well as a sirtuin activator NAD inhibited dopaminergic neurotoxicity of a DNA alkylating agent, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG). Moreover, MNNG-induced increase in acetylation of p53, a representative target of sirtuin deacetylase activity, was suppressed by resveratrol. These results indicate that resveratrol can exert neuroprotective actions in dopaminergic neurons. Either antioxidative activity or sirtuin-activating potential may play an important role in the neuroprotectice actions of resveratrol against different kinds of insults.

Key words: Parkinson's disease; Dopamine neuron; Mitochondrial dysfunction; Neuroinflammation; DNA damage; Oxidative stress

1. Introduction

Selective and progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta is a pathological feature of Parkinson's disease (PD). Accumulating evidence indicates that multiple factors, including genetic and environmental ones, contribute to acceleration of dopaminergic neurodegeneration in this neurological disorder [1]. Particularly, mitochondrial dysfunction has been considered one of the most important factors involved in the pathogenesis of PD [2]. This notion is supported by the finding that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), after metabolic conversion to 1-methyl-4-phenyl pyridinium (MPP^+), interferes with mitochondrial respiration via inhibition of complex I, thereby triggering dopaminergic neurodegeneration that leads to parkinsonism [3]. Activities of mitochondrial complexes I and IV are reduced in PD patients [4]. Remarkably, several genetic factors recently implicated in familial forms of PD, such as DJ-1 and PINK1, may have relevance to mitochondrial function [2].

Besides mechanisms intrinsic to dopaminergic neurons, cell-to-cell interactions associated with inflammatory reactions may also play an important role in PD pathogenesis [5]. A large number of reactive microglia are found in the substantia nigra of PD brain [6], and experimentally, microglial activation by lipopolysaccharide or by thrombin leads to degeneration of midbrain dopaminergic neurons in vitro [7,8] and in vivo [9]. Overall, strategies to protect dopaminergic neurons from progressive degeneration should have potentials to counteract multiple kinds of insults.

Resveratrol is a naturally occurring polyphenol contained in red wine and other dietary products. Since the discovery of chemopreventive activity against skin cancer [10],

resveratrol has been shown to exhibit a wide range of biological actions including anticarcinogenesis, cardiovascular protection and anti-inflammatory effects. Cellular and molecular bases of these actions remain unresolved, but may involve regulation of the activities and expression levels of enzymes and proteins, regulation of ion channels, as well as antioxidative actions [11]. Importantly, resveratrol activates Sir2-family proteins (sirtuins) categorized into class III histone deacetylases [12]. Sirtuins play pivotal roles in various cellular processes, including gene silencing, DNA repair and life span extension in response to caloric restriction. These functions of sirtuins may be based on the fact that they can deacetylate and regulate diverse families of transcription factors including p53, FOXO, NF-κB and Ku70 [13].

A growing body of evidence suggests that resveratrol provides beneficial influences on neuronal cells [14,15]. For example, resveratrol protects cultured hippocampal neurons from cytotoxicity of nitric oxide (NO) and β-amyloid [16,17], striatal neurons from polyglutamine toxicity [18] and dorsal root ganglion neurons from axon degeneration [19]. Neuroprotective effect of resveratrol in vivo has also been reported for damage induced by kainate [20] and global ischemia [21]. On the other hand, no information is available concerning dopaminergic neurons, although one study demonstrated protective effect of resveratrol against MPP^+ toxicity in PC12 cells [22]. Here we investigated neuroprotective potential of resveratrol against several kinds of neurotoxic insults on dopaminergic neurons maintained in midbrain slice culture.

2. Materials and Methods

2.1. Drugs and chemicals

Unless otherwise indicated, drugs and chemicals were obtained from Nacalai Tesque (Kyoto, Japan). MPP^+, resveratrol, quercetin, thrombin, nicotinamide, sirtinol and NAD were obtained from Sigma-Aldrich Chemicals (St Louis, MO, USA). *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) was obtained form Wako Pure Chemicals (Osaka, Japan).

2.2. Culture preparation and drug treatment

Organotypic midbrain slice cultures were prepared according to the methods described previously [7,8]. Briefly, postnatal 2-3 day-old Wistar rats (Nihon SLC, Shizuoka, Japan) were anesthetized by hypothermia and were decapitated, the brain was removed from the skull and separated into two hemispheres. Coronal midbrain slices (350 μm thick) were prepared under sterile conditions, and transferred onto microporous membranes (six slices per each membrane; Millicell^R-CM, Millipore, Bedford, MA, USA) in 6-well plates. Culture medium, consisting of 50% minimal essential medium/HEPES, 25% Hanks' balanced salt solution and 25% heat-inactivated horse serum (Invitrogen Japan, Tokyo, Japan) supplemented with 6.5 mg/ml glucose, 2 mM L-glutamine and 10 U/ml penicillin-G / 10 μg/ml streptomycin (Invitrogen Japan), was supplied at a volume of 0.76 ml per each well. Culture medium was exchanged with fresh medium on the next day of slice preparation, and thereafter, every two days. Slices were maintained in a 34 °C, 5% CO_2 humidified atmosphere.

At 17-18 days in vitro, slices were exposed to drugs by transfer of culture inserts to

culture wells filled with 0.76 ml of drug-containing serum-free medium. Serum-free medium consisted of 75% minimal essential medium/HEPES and 25% Hank's balanced salt solution supplemented with 6.5 mg/ml glucose, 2 mM L-glutamine and 10 U/ml penicillin-G / 10 μg/ml streptomycin.

2.3. Immunohistochemistry

After drug treatment, cultures were fixed with 4% paraformaldehyde and processed for tyrosine hydroxylase (TH; EC number 1.14.16.2) immunohistochemistry [7]. We used rabbit anti-TH polyclonal antibody (1:500, AB-152, Chemicon International, Temecula, CA, USA) as the primary antibody, and biotinylated anti-rabbit IgG from goat (1:200, Vector Lab., Burlingame, CA, USA) as the secondary antibody. After incubation with the secondary antibody, cultures were treated with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Lab.), and then peroxidase was visualized with diaminobenzidine and H_2O_2. The maximal number of TH-positive dopaminergic neurons in an area of 520×670 μm^2 in individual slices was counted.

Oxidative damage of slice cultures was evaluated by immunohistochemistry for 8-hydroxyguanine [23]. Procedures were essentially the same as those for TH immunohistochemistry except that, prior to application of antibodies, fixed tissues were treated with 10 μg/ml proteinase K (EC number 3.4.21.64; Ambion, Austin, TX, USA) for 25 min. Anti-8-hydroxyguanine monoclonal antibody (1:500, R&D systems, Minneapolis, MN, USA) and biotinylated anti-mouse IgG(H+L) from horse (1:200, Vector Lab.) were used as a primary and a secondary antibody, respectively.

Expression of SIRT1 in dopaminergic neurons was determined by double immunofluorescence. Mouse anti-TH (1:1000, Sigma) and rabbit anti-SIRT1(H-300) (1:200, Santa Cruz Biotech., Santa Cruz, CA, USA) were used as primary antibodies. Alexa Fluor^{R} 568 goat anti-mouse IgG(H+L) (1:400, Molecular Probes, Eugene, OR, USA) was used to detect TH immunoreactivity. Tyramide Signal Amplification kit with Alexa Fluor^{R} 488 (Molecular Probes) was used to detect specific labeling of SIRT1 [24].

2.4. Propidium iodide uptake

Propidium iodide (PI) uptake was used for assessment of overall injury of slice cultures [8]. PI (Wako Pure Chemicals) was applied at 5 μg/ml during drug treatment, and the fluorescence in slices was observed through an inverted fluorescence microscope with a rhodamine filter set. Fluorescence images were captured through a monochrome chilled CCD camera, and stored images were analyzed with NIH image 1.62 software. The averaged signal intensity in an area of 180 × 180 μm^2 was obtained as the fluorescence value of each slice. Fluorescence values were normalized with the intensity of fluorescence in cultures that received 300 μM MPP^+ for 48 h as 100%.

2.5. Nitrite quantification

Amount of NO released during thrombin treatment was quantified as the concentration of nitrite in culture medium by Griess method. One hundred μl of culture supernatants was collected and reacted with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 2.5% phosphoric acid) for 10 min at room temperature, and absorbance of diazonium compound was measured at a wavelength of 540

nm. The absolute level of nitrite was determined with reference to a standard curve obtained with sodium nitrite.

2.6. Measuremen of intracellular levels of reactive oxygen species

After drug treatment, slices were washed with Dulbecco's modified Eagle's medium (Invitrogen) and loaded with 30 μM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes) for 30 min at 34 °C. Images of fluorescence of 2',7'-dichlorofluorescein (DCF), which was produced by oxidation of DCFH within cells, were captured through a monochrome chilled CCD camera. The fluorescence intensity of individual slices was determined in the same manner as that in PI fluorescence experiments.

2.7. Glutathione determination

Glutathione content was measured according to the method described by Elleman [25] with modification. After drug treatment, slices were collected in 10 mM PBS and centrifuged at 7000 g at 4 °C for 10 min. Then the samples were homogenized in 80 μl of 0.01 M HCl, and 20 μl of 5% 5-sulfosalicylic acid dehydrate was added. Lysates were centrifuged at 15000 g at 4 °C for 30 min. To 80 μl of this supernatant, 501.1 μl of PBS containing 0.5 mM EDTA, 65 μl of 4 mM NADPH and 10 μl of 1 mg/ml glutathione reductase (EC number 1.8.1.7) were added and incubated for 15 min at room temperature. After addition of 50 μl of 10 mM 5,5'-dithiobis-(2-nitrobenzoate), the absorbance of the mixture was measured at a wavelength of 412 nm.

2.8. Western blot analysis

After treatment with MNNG and resveratrol for 72 h, slices were harvested and homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.0), 25 mM β-glycerophosphate (Sigma), 2 mM EGTA·2Na, 1% Triton X-100, 1 mM vanadate, 1% aprotinin (Sigma), 1 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol. Samples were mixed with a sample buffer composed of 124 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.02% bromophenol blue and 4% 2-mercaptoethanol. After boiling for 5 min, samples were subjected to 12% SDS-polyacrylamide gel electrophoresis for 70 min, followed by transfer to PVDF membrane (Millipore) for 70 min. Membranes were blocked for at least 1 h by 5% nonfat milk at room temperature and subsequently incubated with rabbit anti-acetylated p53 (1:1000, Upstate) and anti-β-actin (1:50000, Sigma), overnight at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibodies (Vector Lab.) at room temperature for 1 h, bands were detected with enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) and exposed to Fuji X-ray film.

2.9. Statistical analysis

Results are expressed as mean ± S.E.M. Statistical significance was evaluated with one-way ANOVA followed by Student-Newman-Keuls test, and probability values less than 5% were considered significant.

3. Results

3.1. Resveratrol protects dopaminergic neurons from injuries induced by MPP^+, sodium azide

and thrombin

TH immunohistochemistry identified dopaminergic neurons with developed dendrites localized at a confined region in individual cultured midbrain slices (Fig. 1A). We first examined the effect of resveratrol against the cytotoxicity of MPP^+, a mitochodrial complex I inhibitor and a well-known dopaminergic neurotoxin. Application of 30 μM MPP^+ to slice cultures for 48 h induced a significant decrease in the number of viable dopaminergic neurons. In addition, dopaminergic neurons surviving after MPP^+ treatment exhibited atrophy of dendritic processes and shrinkage of cell bodies (Fig. 1B). We also assessed PI uptake into slices as a measure of whole tissue injury. Because dopaminergic neurons comprise only a small subset of midbrain cells in our cultures (less than 3%), levels of PI fluorescence essentially reflect the extent of injury in cells other than dopaminergic neurons [8]. A marked increase in PI uptake into whole slices was observed after MPP^+ treatment (Fig. 1E), suggesting that MPP^+ induced cell death also in non-dopaminergic cell population.

Resveratrol co-applied with 30 μM MPP^+ protected dopaminergic neurons in a concentration-dependent manner (Fig. 1C, D). The effect was marginal at a concentration of 10 μM, and a significant effect was observed with 30 and 100 μM resveratrol. At these concentrations, resveratrol also attenuated whole tissue injury as determined by PI uptake (Fig. 1E). Protective effect similar to that of resveratrol was observed when quercetin, another sirtuin-activating polyphenol, was applied concomitantly with MPP^+ (Fig. 1F, G). At 100 μM, quercetin tended to show greater protection than resveratrol against whole tissue injury.

To determine whether resveratrol can afford dopaminergic neuroprotection against other cytotoxic insults, we next examined cytotoxicity of sodium azide, a mitochondrial complex

IV inhibitor. Sodium azide applied at 200 μM for 24 h caused a prominent decrease in the number of viable dopaminergic neurons and an increase in PI uptake into slices. Resveratrol (30-100 μM) concomitantly applied with sodium azide protected dopaminergic neurons (Fig. 2A) and reduced whole tissue injury (Fig. 2B) in a concentration-dependent manner.

We recently reported that thrombin triggered dopaminergic neurodegeneration via activation of microglia and resultant NO production [8]. We found here that resveratrol was also effective against thrombin cytotoxicity. As expected, thrombin applied to slice cultures at 300 U/ml for 72 h caused a significant decrease in the number of dopaminergic neurons, an increase in PI uptake into slices, and an increase in NO production as reflected by nitrite levels in the medium. Concurrent application of resveratrol (30-100 μM) significantly inhibited thrombin-induced loss of dopaminergic neurons (Fig. 2C). PI uptake into slices (Fig. 2D) and nitrite accumulation in the medium (Fig. 2E) were significantly attenuated by 100 μM resveratrol. Taken together, these results indicate that resveratrol shows neuroprotective effects on dopaminergic neurons undergoing several different kinds of insults.

3.2. Protective effect of resveratrol against MPP^+ cytotoxicity involves antioxidative actions rather than sirtuin-activating properties

Resveratrol and quercetin have the ability of activating sirtuin family of histone deacetylases. If the protective effects of resveratrol and quercetin on MPP^+-induced injury are mediated by sirtuins, these effects would be abrogated by co-treatment with sirtuin inhibitors. Accordingly, we examined the effect of nicotinamide, a sirtuin inhibitor [13], on neuroprotective actions of resveratrol and quercetin. As shown in Fig. 3, protective effects

of resveratrol and quercetin at 100 μM against MPP^+-induced loss of dopaminergic neurons and whole tissue injury were not significantly affected by concurrent application of 0.3 and 3 mM nicotinamide. Sirtinol (100 μM), another sirtuin inhibitor, also showed no effect on the protective action of resveratrol (data not shown). Nicotinamide and sirtinol did not affect MPP^+ cytotoxicity by themselves. These results suggest that sirtuin-activating properties of resveratrol and quercetin play a negligible role in their neuroprotective effects against MPP^+ cytotoxicity.

The primary action of MPP^+ is inhibition of complex I of the mitochondrial respiratory chain. Complex I inhibition leads to an increase in production of reactive oxygen species (ROS), which in turn mediate MPP^+ cytotoxicity [3]. Considering that polyphenol compounds including resveratrol possess antioxidative properties [11,26], we assumed that the protective effect of resveratrol against MPP^+ cytotoxicity was relevant to reduction of oxidative stress. To test this possibility, we measured DCF fluorescence as an indicator of intracellular ROS levels. When loaded with DCFH-DA, slices treated with 20 μM MPP^+ for 48 h showed a clear increase in fluorescence of DCF, an oxidized product of DCFH. Concurrent application of resveratrol (30-100 μM) markedly attenuated MPP^+-induced increase in DCF fluorescence (Fig. 4A). We also measured the content of glutathione, a major endogenous antioxidant, in slice cultures. Treatment of midbrain slice cultures with 20 μM MPP^+ for 48 h resulted in a marked decrease in tissue glutathione content, which was prevented by resveratrol (Fig. 4B).

To further verify that attenuation of oxidative stress is a key factor for neuroprotection by resveratrol, we performed immunohistochemistry for 8-hydroxyguanine. 8-Hydroxyguanine is a representative marker of cellular oxidative damage that reflects oxidative modification in

nuclear and mitochondrial DNA [23]. Many cells within midbrain slices treated with 20 μM MPP^+ for 12 h exhibited increased immunoreactivity for 8-hydroxyguanine, as compared to those in slices without drug treatment. Co-administration of 100 μM resveratrol with MPP^+ prevented the increase in 8-hydroxyguanine-immunopositive signals (Fig. 4C-E).

3.3. Resveratrol protects dopaminergic neurons from insults induced by DNA damage

Having shown that the protective effect of resveratrol against MPP^+ cytotoxicity may be attributable to antioxidative properties, we set out to determine whether sirtuin-activating properties of resveratrol have a chance to play a role in dopaminergic neuroprotection. For this purpose, we utilized MNNG, a DNA alkylating agent. DNA damage triggers activation of enzymes for DNA repair such as poly(ADP-ribose) polymerase-1 (PARP-1; EC number 2.4.2.30), whose over-activation depletes cellular NAD [27], possibly leading to suppression of NAD-dependent cellular processes including sirtuin activity [13]. Treatment of midbrain slice cultures with 1 mM MNNG for 72 h induced a significant decrease in the number of dopaminergic neurons. MNNG also caused an increase in PI uptake into slices, although the degree of the increase was smaller than that induced by other insults such as MPP^+, sodium azide and thrombin. Concurrent application of NAD (100-500 μM) with 1 mM MNNG inhibited MNNG-induced dopaminergic cell death (Fig. 5A) and whole tissue injury (Fig. 5B), suggesting that MNNG neurotoxicity is mediated by depletion of NAD. MNNG cytotoxicity was also suppressed by resveratrol in a concentration-dependent manner (Fig. 5C, D). As to dopaminergic neurons, the protective effect of resveratrol was marginal at 1 μM, and a significant effect was observed at 10 and 100 μM.

Several target proteins for deacetylating activity of sirtuins have been identified [13].

Here we focused on the acetylation levels of p53, a tumor suppressor protein implicated in regulation of cell death and survival [28]. Western blot analysis demonstrated that the level of acetylated p53 increased in response to MNNG treatment in midbrain slice cultures (Fig. 5E). Resveratrol (1-100 μM) concomitantly applied with 1 mM MNNG blocked the increase of acetylated p53 level, suggesting that resveratrol indeed activated sirtuins mediating deacetylation of p53.

Finally, we examined SIRT1 expression in our midbrain slice cultures, by double immunofluorescence for TH and SIRT1. We found that many, though not all, TH-positive neurons displayed diffuse immunoreactivity for SIRT1 (Fig. 5F). SIRT1 immunoreactivity was also present in cells other than TH-positive neurons.

4. Discussion

In this study we evaluated neuroprotective potential of resveratrol on midbrain dopaminergic neurons receiving several types of insults. We first examined cytotoxicity of MPP^+, a representative dopaminergic neurotoxin. MPP^+ applied under the present experimental conditions caused a substantial decrease in surviving dopaminergic neurons, but also a prominent increase in PI uptake into slices indicative of non-specific tissue injury. Indeed, MPP^+ and other dopaminergic neurotoxins were shown to require additional NMDA receptor stimulation to induce selective loss of dopaminergic neurons in organotypic culture consisting of substantia nigra, striatum and cortex [29]. In any case, resveratrol as well as another polyphenol compound, quercetin, was able to attenuate MPP^+ cytotoxicity. These observations are in line with a recent report showing that quercetin suppresses MPP^+

cytotoxicity on dopaminergic neurons in dissociated primary culture [30].

We also demonstrated that resveratrol prevented cytotoxic effects of sodium azide and thrombin, in the same range of concentrations as that required to prevent MPP^+ cytotoxicity. Sodium azide inhibits the activity of complex IV of the mitochondrial respiratory chain [31]. A serine protease thrombin activates microglia, and triggers dopaminergic neurodegeneration via induction of inducible NO synthase and increased NO production [8]. Mitochodrial dysfunction and inflammatory processes associated with glial activation are two major factors involved in PD pathogenesis [2,5]. The fact that resveratrol effectively prevented dopaminergic neurodegeneration induced by these different insults suggests that resveratrol and related compounds are potentially useful in retarding dopaminergic neurodegeneration in PD.

We conclude that sirtuin activation does not play a major role in the protective effect of resveratrol against MPP^+ cytotoxicity, because sirtuin inhibitors such as nicotinamide and sirtinol did not counteract neuroprotection by resveratrol. Instead, we propose that antioxidative actions are responsible for neuroprotection by resveratrol against MPP^+. Inhibition of mitochondrial complex I by MPP^+ results in increased production of ROS, which plays a pivotal role in MPP^+-induced degeneration of dopaminergic neurons [3]. Resveratrol is well known to exert potent antioxidative actions, and has been reported to attenuate ROS accumulation induced by NO donors in hippocampal neurons [16] or by β-amyloid in PC12 cells [32]. Besides its own antioxidative properties, resveratrol may suppress oxidative stress via induction of cellular defense mechanisms against ROS. In PC12 cells, resveratrol activates a transcription factor Nrf2, resulting in upregulation of cellular antioxidative factors such as glutathione and heme oxygenase-1 (EC number 1.4.99.3) [33]. Overall, these

properties of resveratrol are consistent with our present findings that resveratrol prevented ROS accumulation, glutathione depletion and cellular oxidative damage in response to MPP^+.

We did not explore detailed mechanisms of the effect of resveratrol against cytotoxicity of sodium azide and thrombin, but we assume potential involvement of antioxidative actions also in these cases. Application of sodium azide has been shown to increase production of ROS in brain tissues [34]. Concerning thrombin, we previously showed that a peroxynitrite scavenger FeTPPS and an antioxidant *N*-acetylcysteine could prevent dopaminergic neurodegeneration induced by thrombin [8], which suggests that peroxynitrite produced from the reaction of NO with superoxide is responsible for neurotoxicity. Therefore, antioxidative actions of resveratrol are likely to play an important role in dopaminergic neuroprotection against these insults. However, further investigations are required to draw a definitive conclusion, and we do not exclude the possibility that other mechanisms are involved in the protective action. For example, we found here that a high concentration (100 μM) of resveratrol partially suppressed nitrite accumulation in response to thrombin. This might be accomplished by direct scavenging of NO by resveratrol, but may also be mediated by actions of resveratrol on microglia. A recent study has demonstrated that resveratrol prevents β-amyloid neurotoxicity by suppressing microglial signaling [35].

DNA damage may contribute to dopaminergic neurodegeneration in PD, since increases in DNA strand breaks and oxidized guanine nucleotides have been identified in the midbrain of PD patients [36,37]. We showed in the present study that a DNA alkylating agent MNNG induces loss of dopaminergic neurons in slice culture. An important observation is that NAD as well as resveratrol reversed MNNG-induced dopaminergic neurodegeneration. PARP-1, a DNA repair enzyme, is shown to be activated after exposure of astrocyte culture to

MNNG, which results in depletion of cellular NAD and cell death [27]. It is likely that MNNG treatment depletes cellular NAD also in dopaminergic neurons, thereby downregulating the activity of NAD-dependent enzymes including sirtuins.

p53 is one of the important substrates of sirtuins in relation to cell death regulation. Lysine residues at the C-terminal region of p53 are acetylated in response to DNA damage, which leads to stabilization of p53 protein, recruitment of co-activators, and induction of apoptotic cell death [38]. Sirtuins inhibit p53-dependent apoptosis via deacetylation of p53 [28,39]. We indeed found here that increase in the levels of acetylated p53 was suppressed by resveratrol. These results lend further support for the idea that sirtuin activation is responsible for the neuroprotective action of resveratrol against DNA damage. Although we do not have conclusive evidence for direct involvement of p53 activation in MNNG-induced dopaminergic neurodegeneration, several studies implicate p53 as a mediator of dopaminergic cell death induced by other stimuli [40,41]. Yet, other deacetylation targets of sirtuins, such as NF-κB [35,42] and a Bax-associated protein Ku70 [43] might also play an important role in neurodegeneration induced by DNA damage and neuroprotection afforded by resveratrol. These possibilities warrant further investigation. In this context, nuclear localization of NF-κB in midbrain neurons is found in PD patients [44], and sirtuin-dependent deacetylation results in downregulation of NF-κB in several cellular systems [11].

Concentrations of resveratrol required to show neuroprotective actions were in a range of 10 - 100 μM. Other studies on primary neuronal cultures demonstrated effects of resveratrol at a similar concentration range (5 - 50 μM) [16,17,35]. On the other hand, peak serum concentrations of total resveratrol (including sulfated or glucuronide metabolites as well as unmodified resveratrol) are estimated to be ~180 nM at a dose equivalent to two glasses of

red wine [11]. Therefore, dosage of resveratrol obtained from dietary sources alone may not be large enough to exhibit neuroprotective actions. An important point to be considered is that in vitro experiments generally employ high concentrations of neurotoxins to induce neuronal death within a few days. Neuronal death in neurodegenerative disorders, which occurs gradually over many years under continuous presence of low levels of toxic insults, might be prevented by much lower concentrations of resveratrol than those used in the present study in vitro. Moreover, resveratrol given as a medicine rather than as a dietary component may achieve sufficient plasma and brain levels to exert neuroprotective actions. A pharmacologically relevant dose (100 mg/kg body weight) in rodents has been reported to give peak plasma concentrations of ~9 μM for unmodified resveratrol and ~680 μM for total resveratrol [11].

In conclusion, resveratrol protects midbrain dopaminergic neurons against several different types of insults related to PD pathogenesis. Different cellular and molecular mechanisms, such as antioxidative actions and regulation of sirtuin-dependent gene transcription, may be involved in the protective actions of resveratrol under different conditions. Resveratrol may represent a novel seed for neuroprotective drugs aimed at prevention of PD.

Acknowledgements

This study was supported by Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science and The Ministry of Education, Culture, Sports, Science and Technology, Japan. H. S. was supported by 21st Century COE Program "Knowledge

Information Infrastructure for Genome Science".

References

[1] de Lau LML, Breteler MMB. Epidemiology of Parkinson's disease. Lancet Neurol 2006;5:525-35.

[2] Abou-Sleiman PM, Muquit MMK, Wood NW. Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nat Rev Neurosci 2006;7:207-19.

[3] Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron 2003;39:889-909.

[4] Benecke R, Strumper P, Weiss H. Electron transfer complexes I and IV of platelets are abnormal in Parkinson's disease but normal in Parkinson-plus syndromes. Brain 1993;116:1451-63.

[5] Herrera AJ, Tomas-Camardiel M, Venero JL, Cano J, Machado A. Inflammatory process as a determinant factor for the degeneration of substantia nigra dopaminergic neurons. J Neural Transm 2005;112:111-9.

[6] Hirsch EC, Hunot S, Damier P, Faucheux B. Glial cells and inflammation in Parkinson's disease: a role in neurodegeneration? Ann Neurol 1998;44 (Suppl 1):S115-20.

[7] Shibata H, Katsuki H, Nishiwaki M, Kume T, Kaneko S, Akaike A. Lipopolysaccharide-induced dopaminergic cell death in rat midbrain slice cultures: role of inducible nitric oxide synthase and protection by indomethacin. J Neurochem 2003;86:1201-12.

[8] Katsuki H, Okawara M, Shibata H, Kume T, Akaike A. Nitric oxide-producing microglia mediate thrombin-induced degeneration of dopaminergic neurons in rat midbrain slice culture. J Neurochem 2006;97:1232-42.

[9] Carreno-Muller E, Herrera AJ, de Pablos RM, Tomas-Camardiel M, Venero JL, Cano J et al. Thrombin induces *in vivo* degeneration of nigral dopaminergic neurons along with

the activation of microglia. J Neurochem 2003;84:1201-14.

[10] Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275:218-20.

[11] Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the *in vivo* evidence. Nat Rev Drug Discov 2006;5:493-506.

[12] Blander G, Guarente L. The Sir2 family of protein deacetylases. Annu Rev Biochem 2004;73:417-35.

[13] Porcu M, Chiarugi A. The emerging therapeutic potential of sirtuin-interacting drugs: from cell death to lifespan extension. Trends Pharmacol Sci 2005;26:94-103.

[14] Anekonda TS. Resveratrol – a boon for treating Alzheimer's disease? Brain Res Rev 2006;52:316-26.

[15] Dore S. Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity. Neurosignals 2005;14:61-70.

[16] Bastianetto S, Zheng W-H, Quirion R. Neuroprotective abilities of resveratrol and other red wine constituents against nitric oxide-related toxicity in cultured hippocampal neurons. Brit J Pharmacol 2000;131:711-20.

[17] Han Y-S, Zheng W-H, Bastianetto S, Chabot J-G, Quirion R. Neuroprotective effects of resveratrol against β-amyloid-induced neurotoxicity in rat hippocampal neurons: involvement of protein kinase C. Brit J Pharmacol 2004;141:997-1005.

[18] Parker JA, Arango M, Abderrahmane S, Lambert E, Tourette C, Catoire H et al. Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons. Nat Genet 2005;37:349-51.

[19] Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science 2004;305:1010-3.

[20] Wang Q, Yu S, Simonyi A, Rottinghaus G, Sun GY, Sun AY. Resveratrol protects against neurotoxicity induced by kainic acid. Neurochem Res 2004;29:2105-12.

[21] Wang Q, Xu J, Rottinghaus GE, Simonyi A, Lubahn D, Sun GY et al. Resveratrol protects against global ischemic injury in gerbils. Brain Res 2002;958:439-47.

[22] Gelinas S, Martinoli MG. Neuroprotective effect of estradiol and phytoestrogens on MPP^+-induced cytotoxicity in neuronal PC12 cells. J Neurosci Res 2002;70:90-6.

[23] Chen L-J, Gao Y-Q, Li X-J, Shen D-H, Sun F-Y. Melatonin protects against MPTP/MPP^+-induced mitochondrial DNA oxidative damage in vivo and in vitro. J Pineal Res 2005;39:34-42.

[24] Qin W, Yang T, Ho L, Zhao Z, Wang J, Chen L et al. Neuronal SIRT1 activation as a novel mechanism underlying the prevention of Alzheimer disease amyloid neuropathology by calorie restriction. J Biol Chem 2006;281:21745-54.

[25] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70-7.

[26] Orallo F. Comparative studies of the antioxidant effects of *cis*- and *trans*-resveratrol. Curr Med Chem 2006;13:87-98.

[27] Ying W, Alano CC, Garnier P, Swanson RA. NAD^+ as a metabolic link between DNA damage and cell death. J Neurosci Res 2005;79:216-23.

[28] Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK et al. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 2001;107:149-59.

[29] Kress GJ, Reynolds IJ. Dopaminergic neurotoxins require excitotoxic stimulation in organotypic cultures. Neurobiol Dis 2005;20:639-45.

[30] Mercer LD, Kelly BL, Horne MK, Beart PM. Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures. Biochem Pharmacol 2005;69:339-45.

[31] Fosslien E. Mitochondrial medicine – molecular pathology of defective oxidative

phosphorylation. Ann Clin Lab Sci 2001;31:25-67.

[32] Jang J-H, Surh Y-J. Protecitve effect of resveratrol on β-amyloid-induced oxidative PC12 cell death. Free Rad Biol Med 2003;34:1100-10.

[33] Chen C-Y, Jang J-H, Li M-H, Surh Y-J. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. Biochem Biophys Res Commun 2005;331:993-1000.

[34] Smith TS, Bennett JP Jr. Mitochondrial toxins in models of neurodegenerative diseases. I: in vivo brain hydroxyl radical protection during systemic MPTP treatment or following microdialysis infusion of methylpyridinium or azide ions. Brain Res 1997;765:183-8.

[35] Chen J, Zhou Y, Mueller-Steiner S, Chen L-F, Kwon H, Yi S et al. SIRT1 protects against microglia-dependent amyloid-β toxicity through inhibiting NF-κB signaling. J Biol Chem 2005;280:40364-74.

[36] Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD et al. Oxidative DNA damage in the Parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. J Neurochem 1997;69:1196-203.

[37] Hegde ML, Gupta VB, Anitha M, Harikrishna T, Shankar SK, Muthane U et al. Studies on genomic DNA topology and stability in brain regions of Parkinson’s disease. Arch Biochem Biophys 2006;449:143-56.

[38] Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. Oncogene. 1999;18:7644-55.

[39] Smith J. Human Sir2 and the 'silencing' of p53 activity. Trends Cell Biol 2002;12:404-6.

[40] Lee S-J, Kim D-C, Choi B-H, Ha H, Kim K-T. Regulation of p53 by activated protein kinase C-δ during nitric oxide-induced dopaminergic cell death. J Biol Chem 2006;281:2215-24.

[41] Biswas SC, Ryu E, Park C, Malagelada C, Greene LA. Puma and p53 play required roles in death evoked in a cellular model of Parkinson disease. Neurochem Res 2005;30:839-45.

[42] Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA et al. Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J 2004;23:2369-80.

[43] Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 2004;305:390-2.

[44] Hunot S, Brugg B, Ricard D, Michel PP, Muriel MP, Ruberg M et al. Nuclear translocation of NF-κB is increased in dopaminergic neurons of patients with Parkinson disease. Proc Natl Acad Sci USA 1997;94:7531-6.

Figure Legends

Fig. 1. Resveratrol and quercetin prevent MPP^+-induced dopaminergic cell death and whole tissue injury. (A-C) Representive photomicrographs showing TH immunohistochemistry of a slice cultured under control conditions (A), a slice treated with 30 μM MPP^+ for 48 h (B) and a slice treated with 30 μM MPP^+ plus 100 μM resveratrol (resv.) for 48 h (C). Scale bar, 50 μm. (D-G) Effects of resveratrol and quercetin on MPP^+-induced dopaminergic cell death (D and F) and PI uptake (E and G). Resveratrol and quercetin at indicated concentrations were applied concomitantly with 30 μM MPP^+ for 48 h. Number of slices examined for each condition is 5-18. *** $p < 0.001$ vs. control (Cont.); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. MPP^+ alone.

Fig. 2. Resveratrol prevents dopaminergic cell death and whole tissue injury induced by sodium azide (NaN_3) and thrombin. (A and B) Effect of resveratrol on NaN_3-induced dopaminergic cell death (A) and PI uptake (B). Resveratrol at indicated concentrations was applied concomitantly with 200 μM NaN_3 for 24 h. (C-E) Effect of resveratrol on thrombin-induced dopaminergic cell death (C), PI uptake (D) and nitrite increase (E). Resveratrol at indicated concentrations was applied concomitantly with 300 U/ml thrombin for 72 h. Number of slices examined for each condition in A-D is 14-32. The number of culture wells examined for nitrite determination (E) are indicated in parentheses. *** $p < 0.001$ vs. control (Cont.); ## $p < 0.01$, ### $p < 0.001$ vs. NaN_3 or thrombin alone.

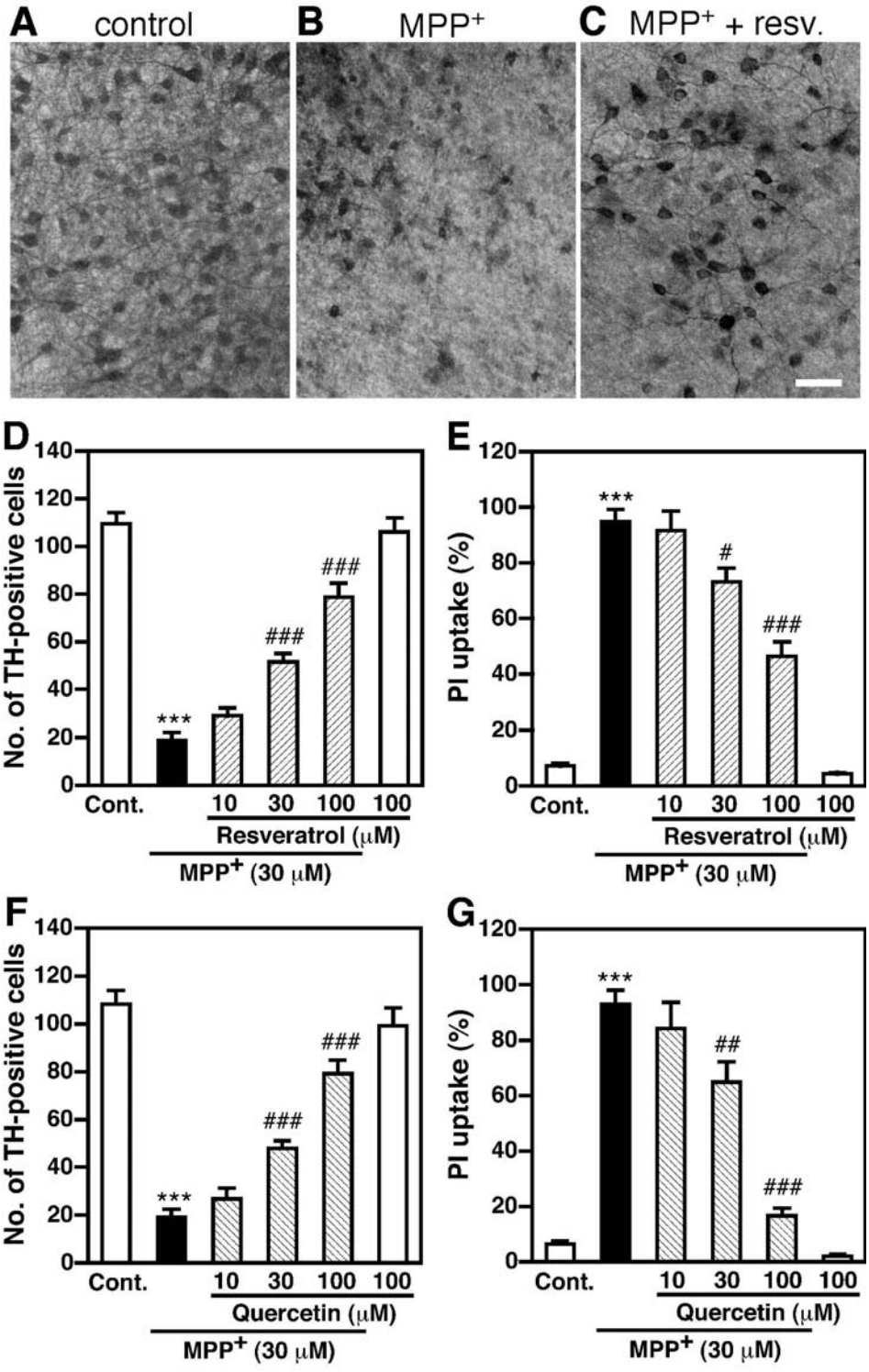
Fig. 3. Sirtuins are not involved in the protective effects of resveratrol and quercetin against MPP^+ cytotoxicity. Effects of nicotinamide (0.3 and 3 mM) on the protective effects of 100 μM resveratrol or quercetin against dopaminergic cell death (A) and PI uptake (B) induced by

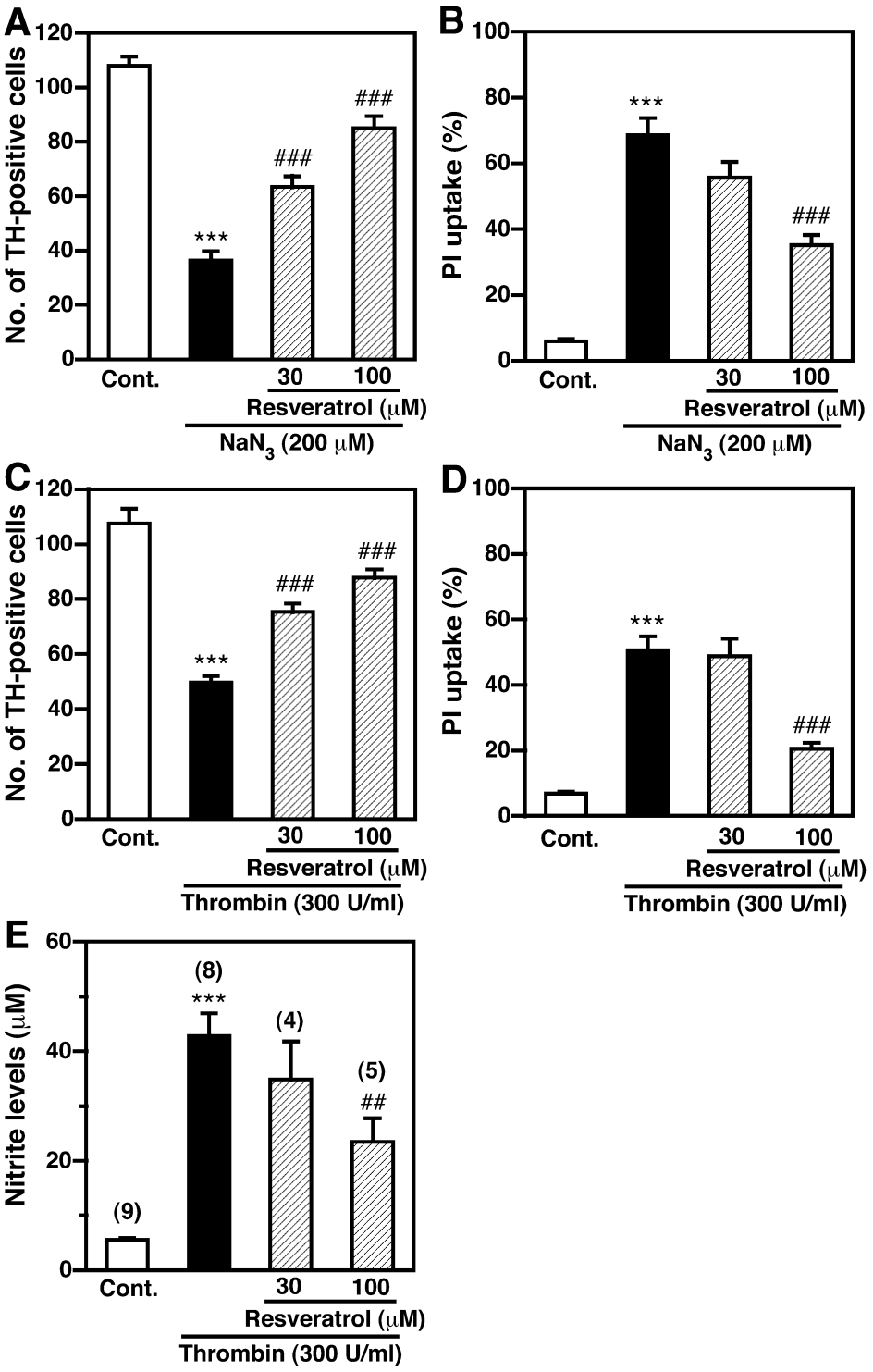
30 μM MPP^+ are shown. All drugs were concomitantly applied to slice cultures for 48 h. Number of slices examined for each condition is 6-30. *** $p < 0.001$ vs. control (Cont.); ### $p < 0.001$ vs. MPP^+ alone.

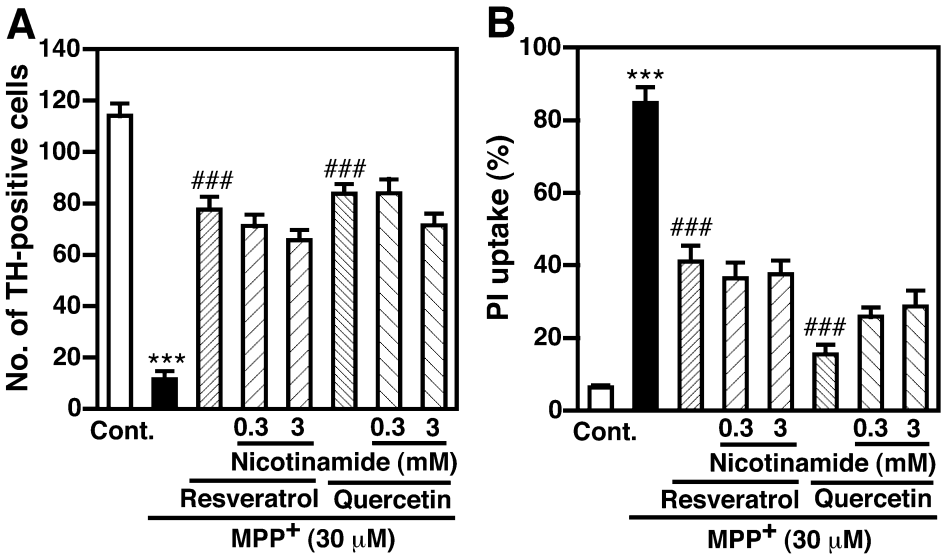
Fig. 4. Antioxidative properties are involved in the protective effect of resveratrol against MPP^+ cytotoxicity. (A and B) Effects of resveratrol on MPP^+-induced increase in DCF fluorescence (A) and decrease in cellular glutathione (B) are shown. Resveratrol at indicated concentrations was applied concomitantly with 20 μM MPP^+ for 48 h. Number of slices examined for each condition in A is 13-16. Number of culture wells examined in B is 4 for each condition. The mean value of glutathione content in control cultures was 36.1 nmol/mg protein. *** $p < 0.001$ vs. control (Cont.); ## $p < 0.01$, ### $p < 0.001$ vs. MPP^+ alone. (C-E) Photomicrographs showing 8-hydroxyguanine immunoreactivity of a slice cultured under control conditions (C), a slice treated with 20 μM MPP^+ for 12 h (D) and a slice treated with 20 μM MPP^+ plus 100 μM resveratrol (resv.) for 12 h (E). Scale bar, 50 μm.

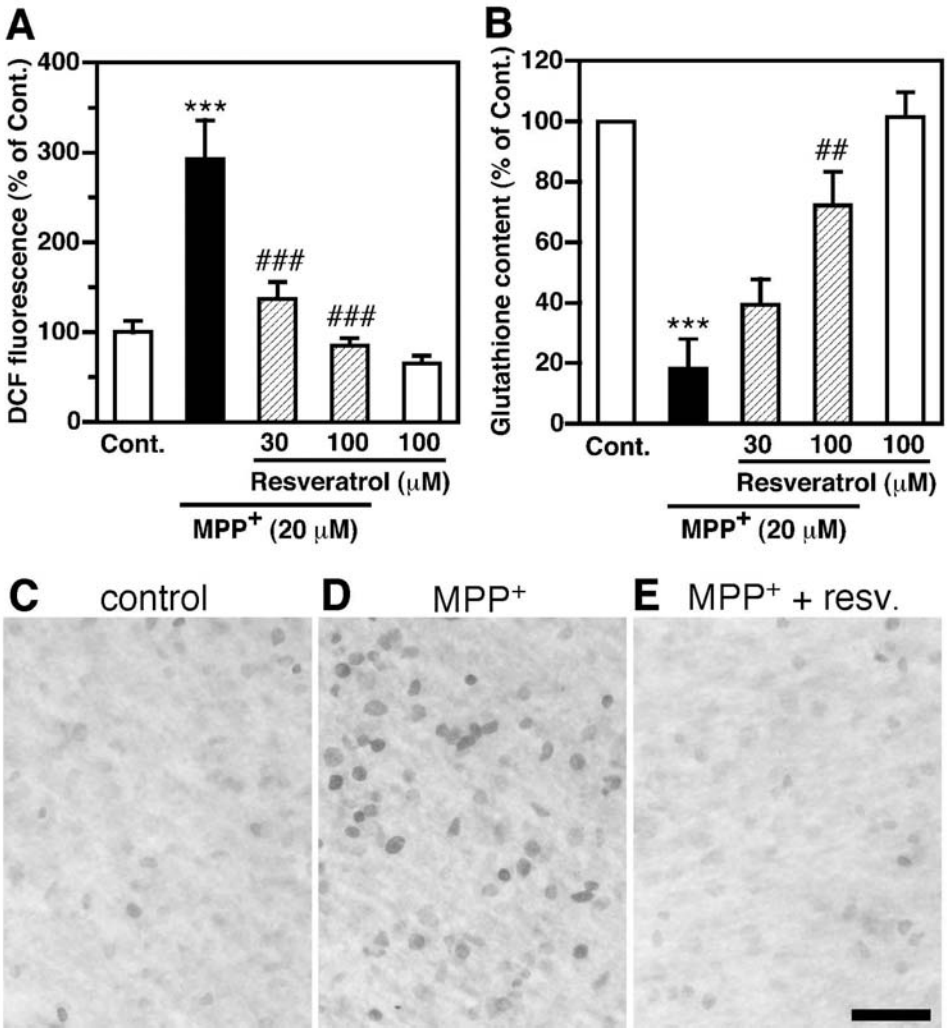
Fig. 5. Effects of NAD and resveratrol on MNNG cytotoxicity. (A and B) NAD inhibits MNNG-induced dopaminergic cell death (A) and PI uptake (B). NAD at indicated concentrations was applied concomitantly with 1 mM MNNG for 72 h. (C and D) Resveratrol inhibits MNNG-induced dopaminergic cell death (C) and PI uptake (D). Resveratrol at indicated concentrations was applied concomitantly with 1 mM MNNG for 72 h. Number of slices examined for each condition is 12-39. *** $p < 0.001$ vs. control (Cont.); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. MNNG alone. (E) Western blot analysis of acetylated p53 (ac-p53) after 72 h treatment of slice cultures with MNNG and/or resveratrol at indicated concentrations. A representative blot of ac-p53 from two independent sets of experiments is shown with that of β-actin, a loading control. (F)

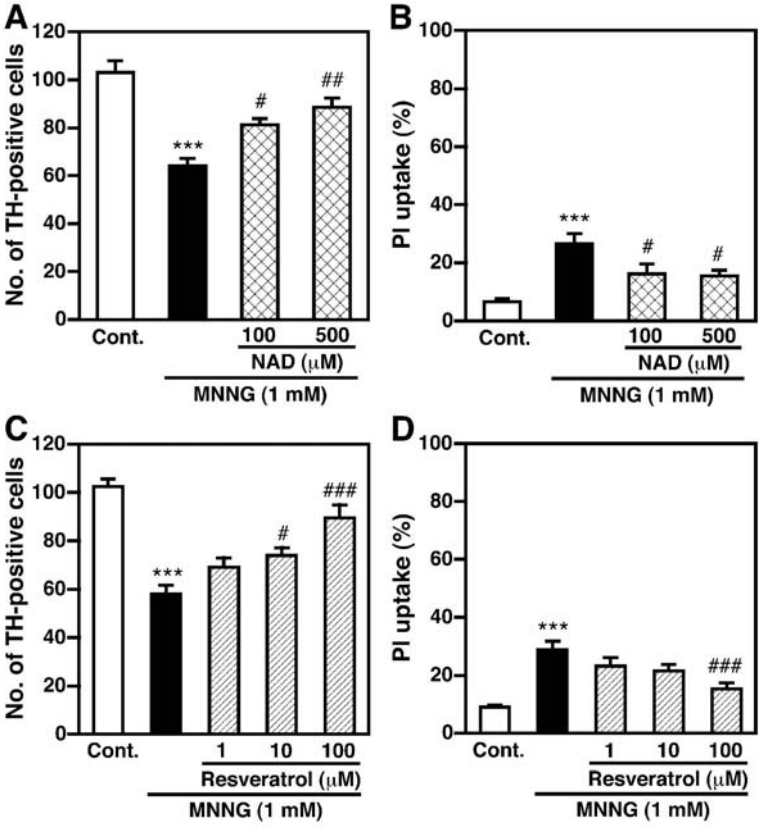
Representative images of double immunofluorescence for TH (red) and SIRT1 (green) in midbrain slice cultures. Arrowheads indicate dopaminergic neurons showing expression of SIRT1. Scale bar, 50 μm.

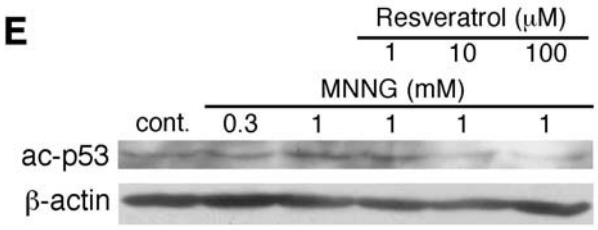

A control
B MPP+
C MPP+ + resv.
D
No. of TH-positive cells
Cont.
10 30 100 100
Resveratrol (μM)
MPP+ (30 μM)
E
PI uptake (%)
Cont.
10 30 100 100
Resveratrol (μM)
MPP+ (30 μM)
F
No. of TH-positive cells
Cont.
10 30 100 100
Quercetin (μM)
MPP+ (30 μM)
G
PI uptake (%)
Cont.
10 30 100 100
Quercetin (μM)
MPP+ (30 μM)

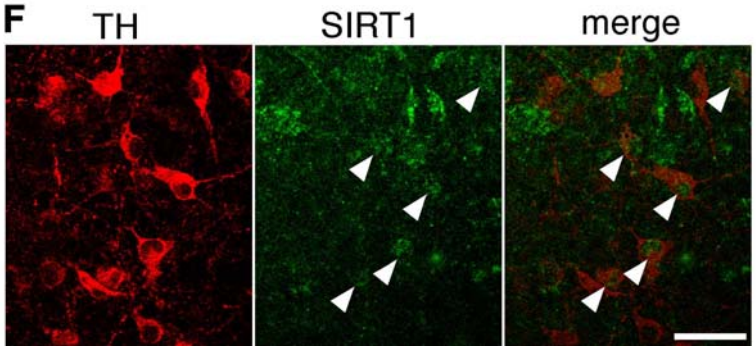

A
No. of TH-positive cells
120
100
80
60
40
20
0
***
###
###
Cont.
30
100
Resveratrol (μM)
NaN3 (200 μM)
B
PI uptake (%)
100
80
60
40
20
0
***
###
Cont.
30
100
Resveratrol (μM)
NaN3 (200 μM)
C
No. of TH-positive cells
120
100
80
60
40
20
0
***
###
###
Cont.
30
100
Resveratrol (μM)
Thrombin (300 U/ml)
D
PI uptake (%)
100
80
60
40
20
0
***
###
Cont.
30
100
Resveratrol (μM)
Thrombin (300 U/ml)
E
Nitrite levels (μM)
60
40
20
0
(9)
(8)
***
(4)
(5)
##
Cont.
30
100
Resveratrol (μM)
Thrombin (300 U/ml)


A
No. of TH-positive cells
140
120
100
80
60
40
20
0
***
###
###
Cont.
0.3
3
0.3
3
Nicotinamide (mM)
Resveratrol
Quercetin
MPP+ (30 μM)
B
PI uptake (%)
100
80
60
40
20
0
***
###
###
Cont.
0.3
3
0.3
3
Nicotinamide (mM)
Resveratrol
Quercetin
MPP+ (30 μM)


A
DCF fluorescence (% of Cont.)
400
300
200
100
0
***
###
###
Cont.
30
100
100
Resveratrol (μM)
MPP+ (20 μM)
B
Glutathione content (% of Cont.)
120
100
80
60
40
20
0
***
##
Cont.
30
100
100
Resveratrol (μM)
MPP+ (20 μM)
C control
D MPP+
E MPP+ + resv.


A
No. of TH-positive cells
120
100
80
60
40
20
0
***
#
##
Cont.
100
500
NAD (μM)
MNNG (1 mM)
B
PI uptake (%)
100
80
60
40
20
0
***
#
#
Cont.
100
500
NAD (μM)
MNNG (1 mM)
C
No. of TH-positive cells
120
100
80
60
40
20
0
***
#
###
Cont.
1
10
100
Resveratrol (μM)
MNNG (1 mM)
D
PI uptake (%)
100
80
60
40
20
0
***
###
Cont.
1
10
100
Resveratrol (μM)
MNNG (1 mM)


E
Resveratrol (μM)
1
10
100
MNNG (mM)
cont.
0.3
1
1
1
1
ac-p53
β-actin


F
TH
SIRT1
merge