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CV-2619 protects cultured astrocytes against reperfusion injury via nerve growth factor production

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Abstract

In this study, we examined the effect of the neuroprotective agent 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone (CV-2619) on reperfusion injury in cultured rat astrocytes after exposure to hydrogen peroxide (H_2O_2)-containing medium. CV-2619 (10 nM to 10 μM) significantly attenuated the reperfusion-induced decrease in cell viability. The compound showed an anti-apoptotic effect in this astrocyte injury model. Antioxidants such as ascorbic acid, α-tocopherol and reduced glutathione also inhibited H_2O_2 exposure-induced cytotoxicity. CV-2619 did not affect the levels of reactive oxygen species, but it increased nerve growth factor (NGF) production. The effect of CV-2619 on H_2O_2 exposure-induced cytotoxicity was blocked by cycloheximide and anti-NGF antibody. The protective effect of CV-2619 was antagonized by the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase inhibitor 2′-amino-3′-methoxyflavone and the phosphatidylinositol-3 kinase inhibitor wortmannin. These findings suggest that the effect of CV-2619 is mediated at least partly by NGF production in astrocytes and that ERK and phosphatidylinositol-3 kinases play a role in the downstream mechanism.

Keywords: CV-2619; NGF (nerve growth factor); Astrocyte; Apoptosis

1. Introduction

2,3-Dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone (CV-2619, previously called idebenone), is a compound with protective efficacy against neurotoxicity and is considered to be useful for treatment of Alzheimer's disease (Bergamasco et al., 1994; Gillis et al., 1994). This compound possibly acts as an antioxidant by blocking lipid peroxidation and other oxidative processes (Suno and Nagaoka 1984a,b; Miyamoto et al., 1989). In addition, CV-2619 increases nerve growth factor (NGF) production in cultured astrocytes (Takeuchi et al., 1990), and oral administration of CV-2619 increases the NGF content in aged and damaged brains (Nitta et al., 1993, 1994). NGF plays an important role in the survival and maintenance of cholinergic neurons in the central nervous system (Gnahn et al., 1983; Hefti et al., 1984; Hefti, 1986). These findings suggest that NGF production is in part, involved in the neuroprotective effect of CV-2619, although the exact mechanism is not known.

Recent studies imply that astrocyte apoptosis may play a role in brain injury (Liu et al., 1997; Petito et al., 1998). We have previously found that Ca^{2+} reperfusion induces delayed death in cultured astrocytes (Matsuda et al., 1996, 1997, 1998; Takuma et al., 1996b), and that reactive oxygen species production is involved in the cell injury, including apoptosis (Takuma et al., 1999). This injury

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model may contribute to clarification of the mechanisms of drugs that ameliorate ischemia–reperfusion-induced brain dysfunction (Takuma et al., 2000). In this paper, we examined the effect of CV-2619 on reperfusion injury in cultured rat astrocytes to clarify the mechanism underlying the protective effect of the compound. The present study demonstrates that CV-2619 inhibits astrocyte apoptosis in a model of reperfusion injury and provides evidence supporting the idea that the effect of CV-2619 is mediated by NGF production.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: fetal calf serum, mouse anti-glial fibrilliary acidic protein antiserum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), α-tocopherol, reduced glutathione (GSH), NGF, anti-NGF 2.5S, anti-fibroblast growth factor-basic (anti-FGF), isolectin B_4 (Biotin labeled), Sigma (St. Louis, MO); NGF E_{max} ™ ImmunoAssay Systems, Promega (Madison, WI); 2′-amino-3′-methoxyflavone (PD98059), Calbiochem (La Jolla, CA); 2′,7′-dichlorofluorescein diacetate (DCF), Molecular Probes (Eugene, OR); wortmannin, Nacalai Tesque (Kyoto, Japan); H_2O_2, ascorbic acid, Wako (Osaka, Japan); Eagle's minimum essential medium (MEM), Nissui Pharmaceutical (Tokyo, Japan); tissue culture ware, Iwaki Glass (Tokyo, Japan); enzyme-linked immunoabsorbant assay (ELISA) plates, Nunc (Roskilde, Denmark). CV-2619 was a gift from Takeda Chemical (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

2.2. Astrocyte culture

Astrocytes were isolated from cerebral cortices of 1-day-old Wistar rats as previously reported (Takuma et al., 1994, 1995, 1996a,b). Briefly, tissue was dissociated with dispase and cultured in MEM containing 10% fetal calf serum and 2 mM of glutamine. Cells were plated in 75-ml tissue culture flasks, divided once upon confluency, and plated in 24-well plastic tissue culture plates or 60-mm plastic tissue culture dishes. The second cultures were grown for 14–20 days in all experiments. The cells were routinely $>95\%$ positive for glial fibrilliary acidic protein, and approximately 2% of the cells were microglia, based on positive isolectin B_4 staining.

2.3. Cell viability

Reperfusion experiments were carried out using confluent astrocytes in fetal calf serum-free medium. Cells were exposed to Ca^{2+}-free or H_2O_2 (100 μM)-containing Earle's solution for 30 min, and then incubated with normal Earle's solution for the indicated times. MTT reduction activity was measured by a colorimetric assay as reported previously (Matsuda et al., 1996; Takuma et al., 1999). MTT reduction activity is expressed as percentage of control.

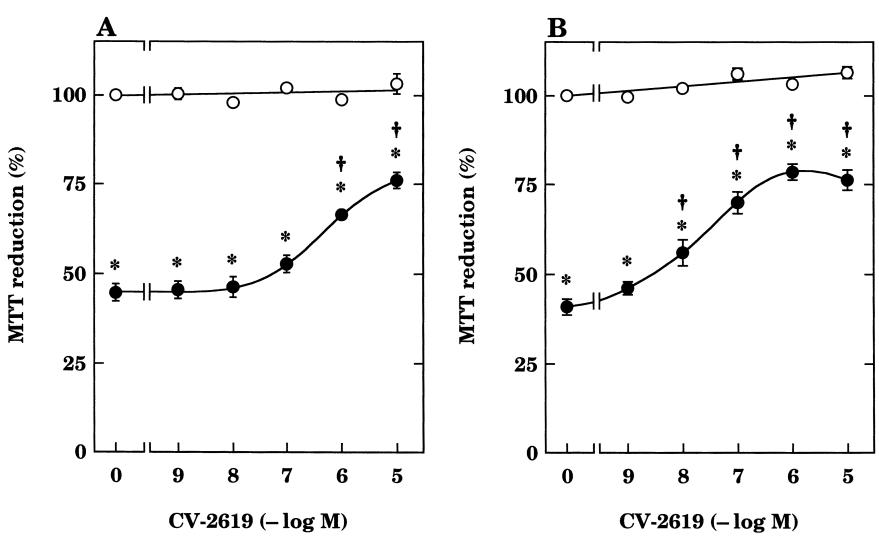

Fig. 1. Effect of CV-2619 on H_2O_2 exposure-induced cell injury in cultured rat astrocytes. Cells were exposed to normal medium (open column) or 100 μM H_2O_2 (hatched column) for 30 min, and then incubated with fresh Earle's solution for 23.5 h. Cell injury was determined by MTT assay. (A) The indicated concentrations of CV-2619 were added after H_2O_2 exposure and were present until assay. (B) The cells were pretreated with CV-2619 at the indicated concentrations in fetal calf serum-containing medium for 7 days, exposed to 100 μM H_2O_2 for 30 min and then incubated with Earle's solution for 23.5 h. Results are means ± S.E. of 9–24 wells obtained from three to six separate experiments. $^{*}P < 0.05$, significant from control; $^{\dagger}P < 0.05$, significant from the values without CV-2619 (Tukey-HSD analysis).

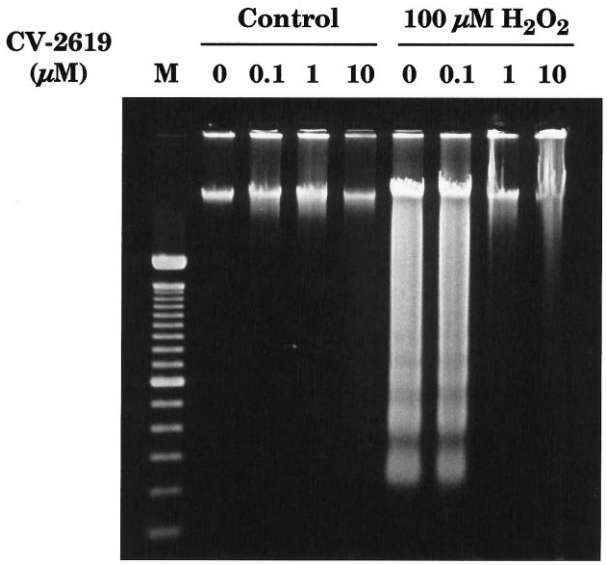

Fig. 2. Effect of CV-2619 on DNA ladder formation induced by reperfusion after H_2O_2 exposure in cultured rat astrocytes. Cells were exposed to normal medium (control) or 100 μM H_2O_2 for 30 min, and then incubated with fresh Earle's solution for 5 days. The indicated concentrations of CV-2619 were present after H_2O_2 exposure. A typical result of two independent experiments is shown (M: 100 bp marker).

2.4. Analysis of DNA ladder

Astrocytes were scraped off using a policeman and collected by centrifugation at 1500 × *g* for 10 min at 4°C. The pellet was suspended in 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5% *N*-lauroylsarcosine and 0.2 mg/ml of proteinase K, and incubated at 37°C overnight. DNA was extracted by phenol/chloroform (1:1; v/v) and precipitated with ethanol. The pellet was dissolved in the Tris–EDTA buffer containing 0.5 mg/ml of RNase A and incubated at 37°C for 30 min to digest RNA. Equal amounts of DNA samples were subjected to 1.8% agarose gel electrophoresis as reported previously (Takuma et al., 1999).

2.5. Hoechst 33342 staining

The cells, plated on a chamber slide, were fixed with 10% formaldehyde and stained with Hoechst 33342 as previously reported (Takuma et al., 1999). An inverted microscope (Olympus, IX70) equipped with a reflected

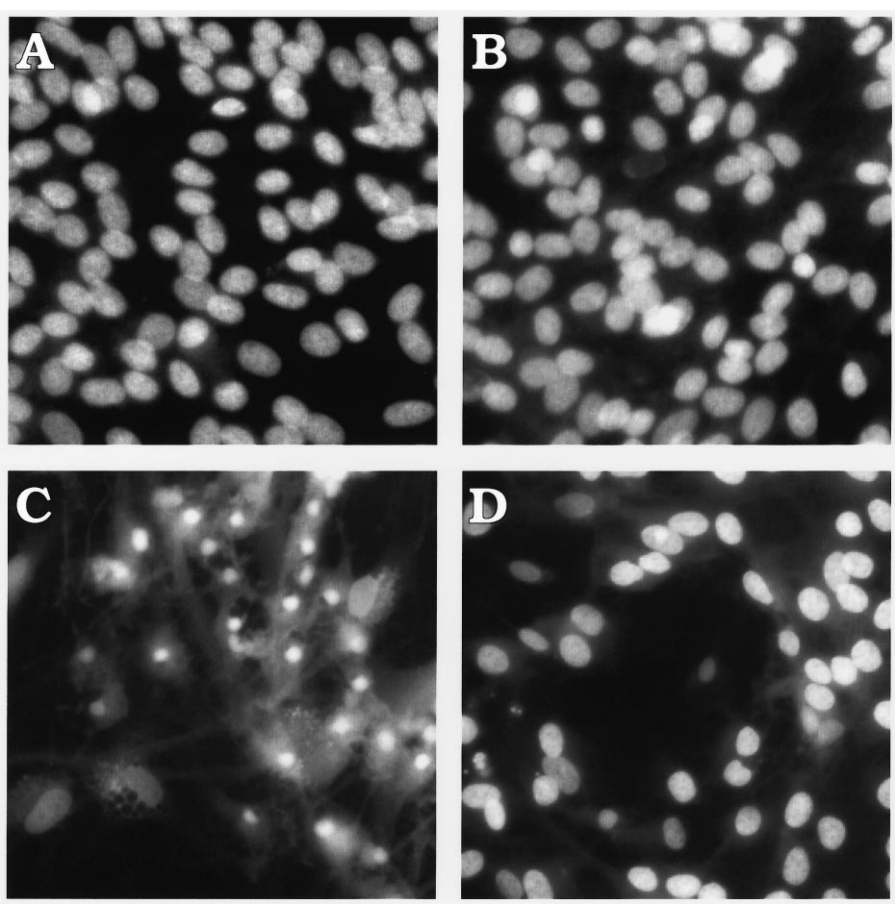

Fig. 3. Effect of CV-2619 on nuclear condensation induced by H_2O_2 exposure in cultured rat astrocytes. Cells were preincubated in the absence (A, B) and presence (C, D) of 100 μM H_2O_2 for 30 min, and incubated with fresh Earle's solution for 3 days. Then, the cells were fixed and stained with Hoechst 33342. CV-2619 (10 μM) was added after H_2O_2 exposure and was present until staining (B, D).

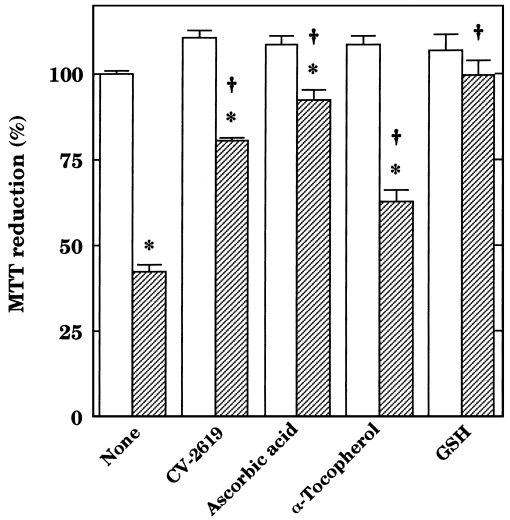

Fig. 4. Effects of CV-2619, ascorbic acid, α-tocopherol and GSH on H_2O_2 exposure-induced cell injury in cultured rat astrocytes. Cells were exposed to normal medium (open column) or 100 μM H_2O_2 (hatched column) for 30 min, and then incubated with fresh Earle's solution for 23.5 h. Cell injury was determined by MTT assay. CV-2619 (10 μM), ascorbic acid (1 mM), α-tocopherol (200 μM) and GSH (1 mM) were added after H_2O_2 exposure and were present for 24 h. Results are means ± S.E. of 9–21 wells obtained from three to seven separate experiments. $^{*}P < 0.05$, significant from control; $^{\dagger}P < 0.05$, significant from the values without drugs (Tukey-HSD analysis).

fluorescence illuminator (Olympus, IX-FLA) and a 40× objective lens was used to visualize individual nuclei.

2.6. Measurement of reactive oxygen species production

Intracellular production of reactive oxygen species was determined using a DCF fluorescent probe (Behl et al., 1994) as reported previously (Takuma et al., 1999). Reactive oxygen species production is expressed as a percentage of control cells.

2.7. Measurement of NGF level

NGF level in cell-conditioned media was determined by a sensitive two-site ELISA according to the manufacturer's instructions. In brief, 96 well, flat-bottomed ELISA plates were coated with anti-NGF polyclonal antibody. The plates containing samples and standards were incubated at room temperature for 6 h on a plate shaker. NGF standards, ranging from 7.8 to 500 pg/ml, were prepared using recombinant human NGF. The captured NGF was reacted first with rat anti-NGF monoclonal antibody, and then with horseradish peroxidase-conjugated anti-rat immunoglobulin G antibody (1:5000).

2.8. Statistics

Statistical analysis of the experimental data was carried out by analysis of variance (ANOVA) followed by post hoc Tukey's honestly significant difference (HSD) multiple comparisons, using SPSS 6.1 for Macintosh.

3. Results

When cultured astrocytes were exposed to H_2O_2-containing medium for 30 min, and then incubated with normal medium, a significant decrease in MTT reduction activity was observed. CV-2619 at 1 and 10 μM inhibited partially reperfusion-induced decreases in MTT reduction activity (Fig. 1A). Furthermore, pretreatment of the cells with CV-2619 in fetal calf serum-containing medium lowered its effective dose: the compound at 10 and 100 nM was effective when astrocytes were pretreated with the compound for 7 days in fetal calf serum-containing medium (Fig. 1B). In most experiments of this study, the compound was added immediately after reperfusion. CV-2619 blocked almost completely DNA ladder formation (Fig. 2) and nuclear condensation (Fig. 3) induced by reperfusion after H_2O_2 exposure. In addition to CV-2619, other anti-oxidants such as ascorbic acid, α-tocopherol and GSH also protected astrocytes from cytotoxicity induced by reperfusion after H_2O_2 exposure (Fig. 4).

CV-2619 did not affect the Ca^{2+} reperfusion-induced increase in reactive oxygen species production: the levels (percentage of control, means ± S.E.M. of 18 wells) of reactive oxygen species at 60 min after Ca^{2+} reperfusion were 196 ± 9 (none), 193 ± 5 (1 μM CV-2619) and 177 ± 5 (10 μM CV-2619). On the other hand, CV-2619 increased the NGF level in astrocytes in a dose-dependent manner (Fig. 5). The CV-2619-induced increase in NGF level was observed in cells pre-exposed to H_2O_2-containing medium for 30 min: NGF levels (pg/ml, means ±

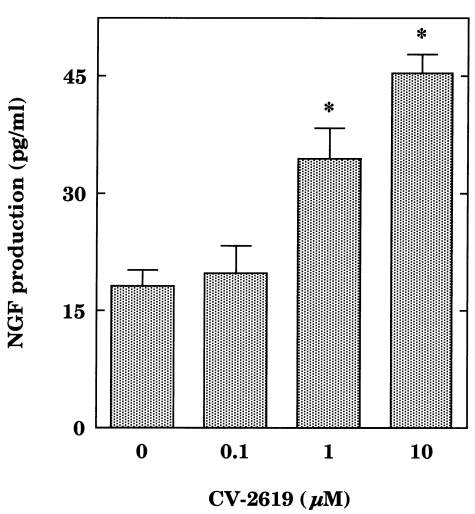

Fig. 5. Effect of CV-2619 on NGF synthesis in cultured rat astrocytes. Cells were treated with the indicated concentrations of CV-2619 for 24 h. Results are means ± S.E. of 6–18 wells obtained from two to six separate experiments. $^{*}P < 0.05$, significant from control (Tukey-HSD analysis).

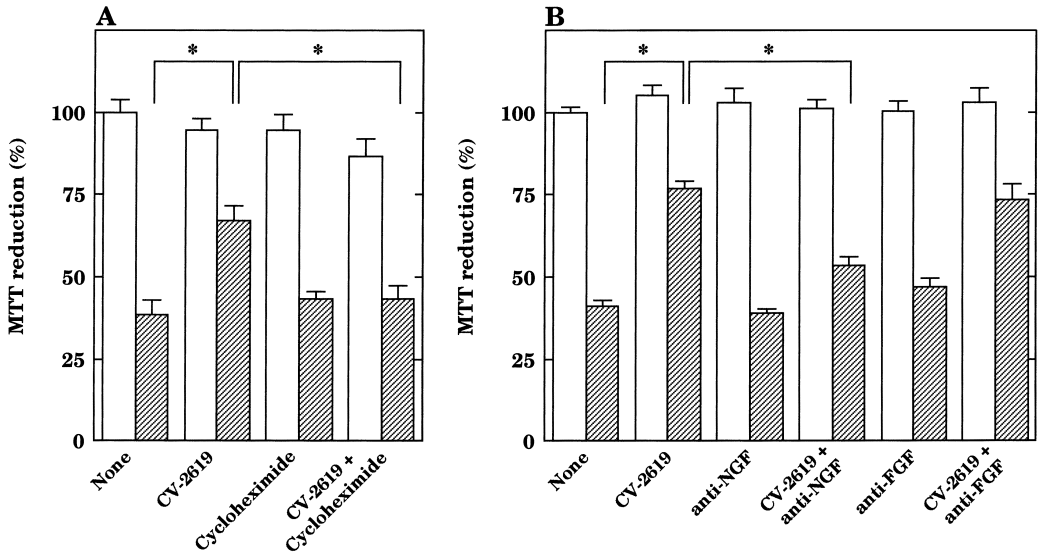

Fig. 6. Effects of cycloheximide, anti-NGF and anti-FGF on protection by CV-2619 against H_2O_2 exposure-induced cell injury in cultured rat astrocytes. Cells were exposed to normal medium (open column) or 100 μM H_2O_2 (hatched column) for 30 min, and then incubated with fresh Earle's solution for 23.5 h. Drugs (CV-2619, 10 μM; cycloheximide, 3 μg/ml; anti-NGF, 1:500; anti-FGF, 1:500) were added after H_2O_2 exposure and present for 24 h. Results are means ± S.E. of 9–27 wells obtained from three to nine separate experiments. $^*P < 0.05$, significant from the values of CV-2619 alone (Tukey-HSD analysis).

S.E.M. of six determinations) were 13.4 ± 2.6 (control) and 34.9 ± 3.7 (10 μM CV-2619, $P < 0.05$). Furthermore, the effect of CV-2619 on MTT reduction activity was antagonized by the protein synthesis inhibitor cycloheximide and by the anti-NGF antibody, but not by the anti-FGF antibody (Fig. 6). When astrocytes were pre-treated with CV-2619 and anti-NGF antibody, the protective effect was not observed (data not shown). The effect of CV-2619 on the decrease in MTT reduction activity induced by H_2O_2 exposure/reperfusion was blocked by the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase inhibitor PD98059 and the phosphatidylinositol-3 kinase inhibitor wortmannin (Fig. 7).

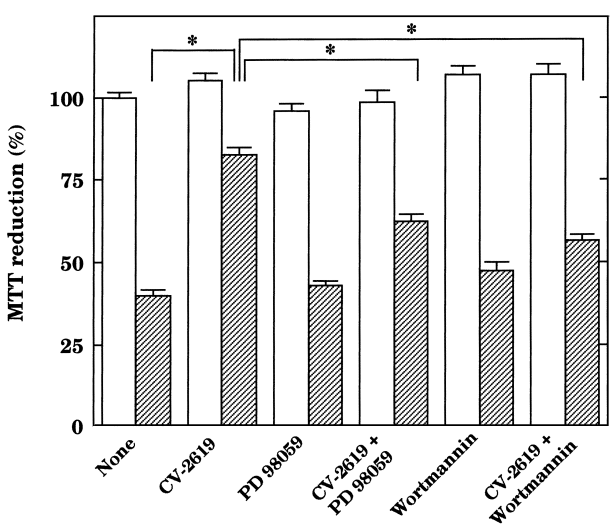

Fig. 7. Effects of PD98059 and wortmannin on protection by CV-2619 against H_2O_2 exposure-induced cell injury in cultured rat astrocytes. Cells were exposed to normal medium (open column) or 100 μM H_2O_2 (hatched column) for 30 min, and then incubated with fresh Earle's solution for 23.5 h. CV-2619 (10 μM) and wortmannin (100 nM) were present after H_2O_2 exposure. PD98059 (100 μM) was added 30 min before H_2O_2 exposure and was present until assay. Results are means ± S.E. of 9–18 wells obtained from three to six separate experiments. $^*P < 0.05$, significant from the values of CV-2619 alone (Tukey-HSD analysis).

4. Discussion

CV-2619 reduces neurological damage after ischemia–reperfusion in an in vivo model (Nagaoka et al., 1989a,b) and protects neurons against β-amyloid-induced neurotoxicity (Hirai et al., 1998). Furthermore, Yamada et al. (1999) have recently reported that oral CV-2619 prevents learning and memory deficits caused by β-amyloid. These studies suggest that CV-2619 is a useful drug for treatment of Alzheimer's disease, but its exact mechanism is not known. In view of the importance of a neuron–glia interaction in neuronal degeneration (Montgomery, 1994; Liu et al., 1997; Petito et al., 1998), the present study was aimed to examine the effect of CV-2619 on astrocyte injury including apoptosis and to study further the involvement of NGF production in the effect of CV-2619.

Although CV-2619 was also effective in reducing paradoxical Ca^{2+} challenge-induced cell injury (data not shown), this study was focused on H_2O_2-induced cytotoxicity, in view of the involvement of reactive oxygen species in β-amyloid-induced neurotoxicity (Pike et al., 1993; Behl et al., 1994; Hensley et al., 1994; Smith et al., 1995).

We have observed that H_2O_2 exposure-induced astrocyte death is due to apoptosis, as determined by DNA fragmentation (Takuma et al., 1999), nuclear condensation (Takuma et al., 1999) and prevention of death by the caspase-3 inhibitor Ac-DMQD-CHO (unpublished results). The present study showed that CV-2619 attenuated H_2O_2-mediated cell death in cultured astrocytes. The protective effect of CV-2619 was observed in the experiments of DNA fragmentation and Hoechst 33342 staining. These findings suggest that CV-2619 inhibits apoptosis. The anti-apoptotic effect of CV-2619 was also reported in cultured cortical neurons: the compound at 3 μM inhibited homocysteate-induced apoptosis (Ratan et al., 1994). In this study, antioxidants such as ascorbic acid, α-tocopherol and GSH also reduced reperfusion injury in astrocytes. Thus, it is likely that the protective effect of CV-2619 in reperfusion injury is mediated by its antioxidant activity, although the compound does not affect the levels of reactive oxygen species in astrocytes.

Previous studies have shown that CV-2619 stimulates NGF synthesis in vitro (Takeuchi et al., 1990) and in vivo (Nitta et al., 1993, 1994). We also observed that CV-2619 stimulated NGF synthesis in cultured astrocytes. The most important finding of this study is that the protective effect of CV-2619 is almost completely blocked by cycloheximide and anti-NGF. This finding suggests that the protective effect of CV-2619 is mediated at least partly by NGF. Furthermore, we showed that the protective effect of CV-2619 was partially blocked by PD98059 and wortmannin. We have found that NGF protects astrocytes against reperfusion injury and the effect is also blocked by PD98059 and wortmannin (Takuma et al., 2000). Taken together, it is likely that CV-2619 stimulates NGF production and then activates the signal pathways of MAP/ERK kinase and phosphatidylinositol-3 kinase, resulting in protection of cultured astrocytes against reactive oxygen species-mediated cytotoxicity.

Hirai et al. (1998) reported that CV-2619 was effective at 10–1000 nM in protecting cultured hippocampal neurons against β-amyloid-induced neurotoxicity. In their experiments, the cells were treated with CV-2619 for 4–5 days. When astrocytes were pretreated with CV-2619 for 7 days, the compound at 10 and 100 nM also protected astrocytes against reperfusion injury. We found that the effect of pretreatment with CV-2619 was also blocked by anti-NGF (data not shown). This suggests that CV-2619 at a low dose also produces NGF. The effectiveness of a low dose of NGF may be explained by an autocrine mechanism, as suggested by Zimmermann et al. (1994). Although the exact mechanism for the effect of pretreatment of CV-2619 is not known, it is likely that the compound at low doses acts on astrocytes as well as neurons as a survival factor. It should be noted that NGF produced in astrocytes in response to CV-2619 not only promotes neuronal survival but also acts on astrocytes. In addition, NGF is considered to be useful for the treatment for Alzheimer's disease (Olson, 1993). Thus, we speculate that the functional change in astrocytes may contribute to the in vivo pharmacological effect of CV-2619.

In conclusion, we have demonstrated that CV-2619 protects astrocytes against cytotoxicity in an in vitro reperfusion injury model and suggest that the effect is mediated by not only its antioxidant activity but also NGF production.

Acknowledgements

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