Amlodipine and Carvedilol Prevent Cytotoxicity in Cortical Neurons Isolated from Stroke-Prone Spontaneously Hypertensive Rats

Kazuo YAMAGATA, Shizuko ICHINOSE**, and Motoki TAGAMI*

We previously reported that vitamin E prevents apoptosis in neurons during cerebral ischemia and reperfusion in stroke-prone spontaneously hypertensive rats (SHRSP). In this paper, we analyzed the effects of antihypertensives as well as vitamin E, which were added to neuron cultures after reoxygenation (20% O2) following hypoxia (1% O2). When added after hypoxia before reoxygenation, vitamin E conferred significant protection to neuronal cells. It was also shown that vitamin E conferred complete protection from neuronal cell death when added hypoxia and again before reoxygenation. At higher concentrations of vitamin E, strong neuroprotection was observed. Moreover, we verified that pretreatment with either amlodipine, carvedilol or dipyridamole consistently prevented cell death during hypoxia and reoxygenation (H/R). On the other hand, nilvadipine, a dihydropyridine-type calcium entry blocker, had no apparent effect on neuroprotection during H/R. The order of neuroprotective potency was vitamin E > dipyridamole > carvedilol > amlodipine > nilvadipine. In parallel experiments, we examined whether these antihypertensive agents were more effective when combined with vitamin E and dipyridamole. The results suggested that in our in vitro model system, antioxidants were the most important agents for the reduction of oxygen-free radical damage in cortical neurons. These findings suggest that amlodipine and carvedilol, with their antioxidant properties and antihypertensive activity, would be useful to inhibit neuronal cell death in the treatment of cerebrovascular stroke and neurodegenerative diseases in hypertensive patients. (Hypertens Res 2004; 27: 271–282)

Key Words: antioxidant, antihypertensive, hypoxia, neuron, stroke-prone spontaneously hypertensive rats

Introduction

Stroke-prone spontaneously hypertensive rats (SHRSP) develop severe hypertension, and more than 95% of them die of cerebral stroke, making them widely used as models for experimental cerebral ischemia (1). In SHRSP, a cerebral ischemia of 20 min duration induces the release of large amounts of glutamate, which causes delayed neuronal death in the CA1 region of the hippocampus (2). We demonstrated that cerebral ischemia followed by reperfusion significantly increased the number of apoptotic neurons in SHRSP, although pretreatments using vitamin E reduced this number (3, 4). Furthermore, showed that vitamin E conferred similar protection against hypertension and cerebral thrombogenesis in SHRSP (5). SHRSP produce more greater concentrations of hydroxyl radicals than Wistar Kyoto rats (WKY) and thus are highly susceptible to neuronal damage (6). It is therefore possible that antioxidants effectively capture hydroxyl radicals produced during hypoxia and reperfusion, thereby preventing neuronal damage in SHRSP. Horakova et al. (7) substantiated the hypothesis that oxygen-free radical are involved in reoxygenation injury in hippocampal. They confirmed that lipid peroxidation was induced and that antioxi-
dants inhibited the injury. McIntosh et al. (8) pointed out that there was increased sensitivity to reactive oxygen species in the temporal cortex of patients with Alzheimer’s disease. This sensitivity may contribute to the pathogenesis of the disease.

Amlodipine significantly inhibited serum-induced increases in cholesterol content and cell-proliferative activity in cultured human cells (9). Another report revealed novel mechanisms in the antiproliferative effects of amlodipine in vascular smooth muscle cells (VSMC) from SHR. Amlodipine inhibited DNA synthesis and proliferation, and expression of platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), and basic fibroblast growth factor (bFGF) mRNAs in VSMC from SHR (10). Other reports demonstrated an association between increased cholesterol levels and lipid peroxidation in the development of atherosclerosis, and showed antiatherogenic effects of amlodipine and vitamin E in an atherosclerotic rabbit model (11). Correspondingly, carvedilol, a multi-functional antihypertensive agent, has been shown to decrease free radicals and inhibit lipid peroxidation in humans (12). Pretreatment with carvedilol was sufficient to prevent vascular smooth muscle proliferation and migration and neointimal formation following vascular injury (13–15). Furthermore, at clinically realistic doses, dipyridamole has a concentration-dependent antioxidant effect in human erythrocytes (16), protects membranes from oxidation and maintains the antioxidant power of erythrocytes. In addition, nilvadipine, a calcium antagonist, has been shown to have antioxidant activity in vivo (17). In the same study, nilvadipine was shown to protect low density lipoprotein (LDL) cholesterol from in vivo oxidation in hypertensive patients at risk of developing atherosclerosis.

The aim of this study was to determine whether antihypertensives which have antioxidative and neuroprotective effects can prevent ischemic neuronal death in SHRSNP during hypoxia and reoxygenation (H/R). To assess the neuroprotective activity, we morphologically analyzed the effects of various antihypertensives (amlodipine, carvedilol, dipyridamole, and nilvadipine) as well as vitamin E, added to neuronal cultures after 3 h of reoxygenation (20% O₂) following 24 h of hypoxia (1% O₂). Furthermore, we examined whether neuronal damage is minimized by combinations of these antihypertensives and vitamin E.

Methods

Cell Culture

We cultured neurons from fetal brains which were not influenced by blood pressure. Primary dissociated neurons were prepared from fetal brains (15 days of gestation) as described previously (18). The brains were then dissected, and all blood vessels and pia mater were removed. The brains were minced into 2–3 mm pieces, which were then washed in phosphate-buffered saline (PBS) and placed in PBS containing 200 µg/ml of streptomycin and 1 mg/ml of ceftazidime. The brain matter was treated with 0.25% (w/v) trypsin at 37°C for 10 min and digested with papain (0.15 U/ml; Funakoshi, Tokyo, Japan), 0.02% (w/v) l-cystein monohydrochloride (Sigma Chemical Co., Tokyo, Japan), 0.02% (w/v) bovine serum albumin (Sigma) and 0.5% (w/v) glucose in PBS at 37°C for 10 min. The dissociated cells were collected by centrifugation and resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan). After a gentle mechanical trituration through a siliconized Pasteur pipette, the cells were filtered through a sterile lens paper filter. The cells in the filtrate were collected by centrifugation, resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco-BRL Life Technologies, Tokyo, Japan), and then plated on a poly-L-lysine-coated 24-well culture plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at a density of 3–4 × 10⁴ cells per well. This point in the experiment was designated as day 0. Forty-eight hours after plating, the cells were treated with 40 µg/ml of 5-fluoro-2-deoxyuridine (Sigma) to prevent the proliferation of nonneuronal cells. The cells were maintained in DMEM with 5% FBS + 5% horse serum (Gibco-BRL Life Technologies) in a 5% CO₂ humidified atmosphere at 37°C. The medium was changed every 2 days. The study was started when nerve fibers and dendrites were well-developed.

Treatment of Cultures

When neurons in culture become mature (days 5–6), they were incubated in 1% O₂, 94% N₂, and 5% CO₂ (hypoxia) for 24 h. After hypoxic culture, they were maintained in air (about 20% O₂) and 5% CO₂ (reoxygenation) for 3 h. In addition, mature neurons (days 5–6) were cultivated in air (about 20% O₂) and 5% CO₂ for 6 to 48 h to serve as a control. We added 0.1 to 100 µmol/l of antihypertensives to the neuronal cells. Immediately afterwards, we lowered the oxygen concentration and maintained the cells in a humidified atmosphere of 1% O₂, 94% N₂, and 5% CO₂ at 37°C for 24 h (hypoxia). When hypoxic cultures were finished, we again added the agent and incubated the cells in air (about 20% O₂) and 5% CO₂ in a humidified atmosphere at 37°C for 3 h. Amlodipine was provided by Pfizer Pharmaceutical Inc. (Sandwich, UK). Calvedilol was provided by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Dipyridamole was purchased from Sigma. Nilvadipine was provided by Funakoshi Pharmaceutical Co. (Tokyo, Japan). Vitamin E (α-DL-tocopherol) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Amlodipine, nilvadipine and vitamin E were dissolved in DMEM supplemented with 5% FBS + 5% horse serum, with the final concentration of ethanol being less than 0.01%. Calvedilol and dipyridamole were dissolved in DMEM supplemented with 5% FBS + 5% horse serum, with the final concentration of dimethylsulfoxide (DMSO, Sigma) being less than 0.01%.
Evaluation of Neuronal Cell Death

Viable and nonviable cells were counted under an electron microscope by two of the authors as reported previously (3). In addition, we determined that the cells were intact, necrotic, or apoptotic by criteria described previously (3).

Electron Microscopic Examination

The cultures were terminated by fixing the cells with 1.25% glutaraldehyde (Wako) and 1% paraformaldehyde (Wako) in 0.1 mol/l PBS for 30 min. The cells were washed overnight at 4°C in the same buffer, and post-fixed with 2% OsO4 (Wako) buffered with 0.1 mol/l PBS for 1 h. The cells were dehydrated in a graded series of ethanol concentrations and embedded in Epon 812 (Wako). Ultrathin sections were double-stained with uranyl acetate and lead citrate for electron microscopic examinations. We performed at least 3 experiments per cultivating condition; for each experiment, we examined at least 3,000 neurons in each of 3 wells (diameter 10 mm). Consequently, we obtained data on 27,000 (3 × 3 × 3,000) neurons per condition.

Statistical Analysis

Data are presented as means ± SD. The significance was determined using Fisher’s Protected Least Significance Difference (PLSD) method following an analysis of variance (ANOVA).

Results

Effects of Vitamin E on the Death of Cultured Neurons during Hypoxia and Reoxygenation

Primary dissociated neurons were prepared from SHRSP. Once the neurons in culture had matured (days 5–6, about 20% O2), they were incubated in 1% O2 for 24 h. When the hypoxic cultures were finished, we maintained the cells in 20% O2 for 3 h. After hypoxia for 24 h, the percentage of dead neuronal cells from SHRSP was 7.8 ± 1.3% (n = 7) (Fig. 1). Similarly, the rate of cell death was 5.1 ± 1.3% (n = 7) when vitamin E (100 µg/ml) was added (p < 0.05). On the other hand, after 3 h of reoxygenation following 24 h of hypoxia, the rate of neuronal cell death from SHRSP without vitamin E was 71.1 ± 9.2% (n = 7; data not shown in this figure).
Effects of Antihypertensives on the Death of Cultured Neurons during Hypoxia and Reoxygenation

Neuronal cells isolated from SHRSP were incubated in 1% O₂ for 24 h. When the hypoxic cultures were ready, we maintained the cells in 20% O₂ for 3 h. All antihypertensives were added before both hypoxia and reoxygenation. After the H/R, the percentage of dead neuronal cells from SHRSP without antihypertensives was 71.1 ± 9.2% (n = 7) (Fig. 2).

In contrast, vitamin E (100 μg/ml) reduced the death of SHRSP neurons exposed to 24 h of hypoxia and 1.5 and 3 h of reoxygenation. The rates of cell death were 24.4 ± 4.1% and 30.6 ± 5.3%, respectively (n = 7). Moreover, neuronal cell death totally ceased when we added vitamin E during reoxygenation in all of the hypoxic conditions (p < 0.0001). The rates of cell death were 5.1 ± 1.3% and 10.6 ± 3.8% (n = 7), respectively, on addition of vitamin E (100 μg/ml).

In contrast, amlodipine inhibited the death of SHRSP neurons exposed to 24 h of hypoxia and 3 h of reoxygenation at 20, 10, 5, 1, and 0.5 μmol/l. The rates of cell death were 37.0 ± 8.7%, 36.7 ± 12.3%, 31.4 ± 11.5%, 39.7 ± 9.2%, and 49.7 ± 7.7% (p < 0.0001, n = 7), respectively (Fig. 2). The maximal inhibition of neuronal cell death was observed at 5 μmol/l (percentage of dead cells: 31.4 ± 11.5%, n = 7).

As shown in Fig. 3, carvedilol inhibited the death of SHRSP neurons exposed to H/R at 20, 10, 5, 1, 0.5 and 0.1 μmol/l. The rates of cell death were 29.6 ± 7.1%, 25.9 ± 5.3%, 34.6 ± 8.6%, 37.0 ± 6.5%, 48.3 ± 5.1%, and 58.3 ± 7.8% (p < 0.0001, n = 7), respectively. The maximal inhibition of neuronal cell death was observed at 10 μmol/l (percentage of dead cells: 25.9 ± 5.3%, n = 7). Likewise, dipyridamole inhibited the death of SHRSP neurons exposed to H/R at 20, 10, 5, 1, 0.5 and 0.1 μmol/l (Fig. 4). The rates of cell death were 22.6 ± 4.5%, 20.0 ± 6.5%, 20.3 ± 5.5%, 25.9 ± 5.9%, 26.1 ± 9.0%, and 44.7 ± 7.1% (p < 0.0001, n = 7), respectively. Strong inhibition of neuronal cell death by dipyridamole was observed at 10 and 5 μmol/l. However, at 30 and 40 μmol/l dipyridamole, crystallization occurred. On the other hand, nilvadipine had no apparent effect on neuroprotection during H/R at 50 (73.0 ± 6.8%), 20 (71.4 ± 5.5%), 10 (71.6 ± 7.7%), 5 (75.8 ± 4.8%), and 1 μmol/l (74.2 ± 8.3%) (data not shown).
Effects of Amlodipine or Carvedilol and Vitamin E on the Death of Cultured Neurons during Hypoxia and Reoxygenation

We further examined the effects of co-treatment with vitamin E on the inhibition by amlopidine or carvedilol. As shown in Fig. 6, the rate of neuronal cell death after H/R with vitamin E (100 μg/ml) alone was 10.6 ± 3.9%, (n = 7). This value is consistent with the data reported previously (3). The rate of neuronal cell death using vitamin E alone (10.6 ± 3.9%, n = 7) was smaller (p < 0.01) than that with amlopidine alone (31.4 ± 11.5%) and also smaller than that with carvedilol alone (25.9 ± 5.3%). The rate for amlopidine (5 μmol/l) in combination with vitamin E was 9.9 ± 2.9% (n = 7). The rate of cell death with amlopidine alone (31.4 ± 11.5%) was markedly greater (p < 0.01) than that with the combination of vitamin E and amlopidine. The effect of combination treatment with vitamin E and carvedilol was similarly striking. The percentage of dead cells for treatment with vitamin E and carvedilol was 10.7 ± 2.8% (n = 7), which was lower (p < 0.01) than that for carvedilol alone. However, the effect of co-treatment with vitamin E was not confirmed. We also examined the effects of co-treatment with low dose of vitamin E on the inhibition by amlopidine or carvedilol. We previously reported that the minimal concentration of vitamin E needed to inhibit neuronal death at H/R was 10 μmol/l (3). In the present study, at 10 μmol/l of vitamin E, the neuronal death rate was slightly lower than that in the control (Figs. 7, 8). This finding was consistent with that in the aforementioned study (3). However, the synergistic effect of co-treatment of amlopidine and carvedilol (0.1, 1 and 10 μmol/l) with vitamin E at minimal inhibitory concentration was not confirmed.

Comparison of the Effects of Various Antihypertensive and Vitamin E on the Death of Cultured Neurons during Hypoxia and Reoxygenation

We compared the inhibition of cell death in cortical neurons by applying vitamin E (100 μg/ml) and various antihypertensives (amlopidine, carvedilol, diprydamole and nilvadipine) after 24 h of hypoxia and 3 h of reoxygenation at the concentration of maximal inhibition (Fig. 9A). All antihypertensives were added before both hypoxia and reoxygenation. The inhibition of cell death in cortical neurons caused by vitamin E was significantly higher (p < 0.01) than that due to amlopidine (5 μmol/l), carvedilol (10 μmol/l), or diprydamole (10 μmol/l). The order of the neuroprotective effects during H/R was vitamin E > diprydamole > carvedilol > amlopidine > nilvadipine. The percentages of dead cells in the cultures treated with vitamin E, diprydamole, carvedilol, amlopidine and nilvadipine were 10.6 ± 3.9%, 20.0 ± 6.5%, 25.9 ± 5.3%, 31.4 ± 11.5% and 71.6 ± 7.7% (n = 7), respectively. We also examined the possibility that antihypertensives protected cortical neurons isolated from WKY against
Fig. 5. Inhibition of cell death by administration of amlodipine and dipyridamole or carvedilol and dipyridamole. The treatment of cultures was carried out as described in Fig. 2 and the Methods section. The concentrations of amlodipine (Aml or A), carvedilol (Car or C) and dipyridamole (Dip or D) used were 5, 10 and 10 μmol/l, respectively. At the end of the experiment, viable and nonviable cells were counted under an electron microscope by two of the authors as reported previously (3). Neuroprotective effects are expressed as percentages of cell death. Values are the means ± SD (n = 7). n.s., not significant.

Fig. 6. Inhibition of cell death by administration of amlodipine and vitamin E or carvedilol and vitamin E. The treatment of cultures was carried out as described in Fig. 2 and the Methods section. The concentration of amlodipine (Aml) carvedilol (Car) and vitamin E (VE) used were 5, 10 μmol/l and 100 μg/ml, respectively. At the end of the experiment, viable and nonviable cells were counted under an electron microscope by two of the authors as reported previously (3). Neuroprotective effects are expressed as percentages of cell death. Values are the means ± SD (n = 7).
H/R-mediated neurotoxicity when administered at a maximally inhibitory concentration. The percentages of cell death in WKY were 42.3 ± 7.2% (control), 9.4 ± 2.7% (vitamin E), 24.1 ± 4.8% (amlodipine), 20.4 ± 6.1% (carvedilol) (Fig. 9B). Although the percentage of cell death in the control was lower in WKY (42%) than in SHRSP (71%), inhibition of neuronal cell death by vitamin E, amlodipine, and carvedilol was confirmed in WKY. Moreover, the inhibitory activities of these agents were similar to those seen in SHRSP neurons following exposure to H/R.

Electron Microscopic Examination

Finally, we examined the effects of antihypertensives on cell death in cultured SHRSP neurons during 24 h of hypoxia and 3 h of reoxygenation by electron microscopy (Figs. 10 and 11). As shown in Fig. 10B, SHRSP neurons maintained in 1% O₂ for 24 h and then cultured in 20% O₂ for 3 h without any neuroprotective agents were damaged and packed with lipid droplets (LD). Most cell organelles had lost their original structure. Many nuclei had disappeared, and others had become electron-dense and small. Under the same conditions, cells treated with vitamin E (Fig. 10A) were intact. Numerous cell organelles and normal nuclei were observed. In addition, the cells maintained with 10 μmol/l of carvedilol or 5 μmol/l of amlodipine (Fig. 11), were well-preserved, although many LD were observed within the cell bodies (Fig. 11B).

Discussion

Neuronal cell death as a result of apoptosis is associated with cerebrovascular stroke and various neurodegenerative disorders (19). Pharmacological agents that maintain normal intracellular Ca²⁺ levels and inhibit cellular oxidative stress may be effective in blocking abnormal neuronal apoptosis. In this study, we demonstrated the effects of antihypertensives on the death of cultured SHRSP neurons during H/R by electron microscopy. H/R heavily damaged neuronal SHRSP cells. The morphologic changes were consistent with the data we reported previously (3). In the present study, we showed that amlodipine, carvedilol and dipyridamole clearly inhibited cell death in cultured SHRSP neurons during H/R. In addition, we examined the effect of co-treatment with
vitamin E or dipyridamole.

In this study we have shown that neural cell death occurs after the reoxygenation following hypoxia, and not during hypoxia itself (Fig. 1). However, cell death is not completely preventable by only vitamin E (100 µg/ml) treatment during the reoxygenation phase. In addition, we found it necessary to add vitamin E both before hypoxia and reoxygenation to completely prevent neuronal cell death. There is considerable direct and indirect evidence that oxygen-free radicals are generated in the first few minutes after reperfusion and result in cellular damage (20, 21). Recent reports indicate that vitamin E blocks early events induced by 1-methyl-4-phenylpyridinium (MPP+) in cerebellar granule cells (CGCs) (21). Exposure of CGCs to MPP+ results in apoptotic cell death, which is markedly attenuated by co-treatment of CGCs with vitamin E (22). In the present study, we showed that apoptosis was completely prevented by adding vitamin E both before hypoxia and reoxygenation. This result is confirmed the data reported previously (22). In our in vitro model system, vitamin E alone may be insufficient, but the precise mechanisms responsible for these effects remain unclear.

SHRSP neurons are more susceptible than WKY neurons to hypoxia. It has been demonstrated that oxygen radicals heavily damage the cells within the first few minutes after reperfusion, and that antioxidants, including vitamin E, react with the radicals, thereby preventing apoptosis and necrosis (3). Our results suggest that antioxidants, including vitamin E, should be added before both hypoxia and reoxygenation as protection for the neural cells (Fig. 1). We previously examined the effect of allopurinol (a xanthine oxidase (XOD) inhibitor) on neurons in order to clarify the mechanism that causes reperfusion injury (3). In brief, when the hypoxic cultures were finished, we added 1 µmol/l allopurinol to the cells and incubated them in an atmosphere of 20% O2 for 1.5 to 5 h. When this agent was added once before the reperfusion, it did not reduce apoptosis. Furthermore, the apoptosis did not significantly increase when the agent was administered every 90 min during the reperfusion. Our previous report indicated that allopurinol inhibits the XOD activity and reduces the production of oxygen-free radicals, thereby preventing apoptosis. In our in vitro system, vitamin E and allopurinol provided comparable neuroprotection in basal medium (3). XOD is the first free radical-producing substance. Therefore, a major cause of neuronal death in our in vitro system seems to be oxidative stress, which is diminished by antioxidant agents.

Subsequently, in this study we have demonstrated that amlodipine inhibited the death of SHRSP neurons by up to 31.5% (Fig. 2). These findings correspond to the results of a similar study that showed that amlodipine has a more potent neuroprotective effect than neutral Ca2+ channel blockers (nifedipine and nimodipine) in rat CGCs cells (23). Amlodipine exhibited very potent neuroprotective activity in this system, compared with antioxidants and neutral Ca2+ channel blockers (nifedipine and nimodipine). Within its effective pharmacological range (10–100 nm), amlodipine attenuated intracellular neuronal Ca2+ increases elicited by KCl depolarization but did not affect Ca2+ changes triggered by N-methyl-D-aspartate (NMDA) receptor activation. Amlodipine also inhibited free radical-induced damage to lipid con-
Fig. 10. Cortical neurons isolated from SHRSP—effect of vitamin E. A: Neurons maintained in 1% O₂ for 24 h and then cultured in 20% O₂ for 3 h with 100 µg/ml vitamin E. Numerous cell organelles are present within the cell bodies. They are intact. In addition, nuclei (N) are normal. B: Neurons maintained in 1% O₂ for 24 h and then cultured in 20% O₂ for 3 h without any agents. The cells are damaged and packed with lipid droplets (LD) and myeline figures (m). Most cell organelles have lost their original structure. Many nuclei have disappeared, and others (N) have become small and electron-dense.

Fig. 11. Cortical neurons isolated from SHRSP—effect of carvedilol and amlodipine. A: Neurons maintained in 1% O₂ for 24 h and then cultured in 20% O₂ for 3 h with 10 µmol/l carvedilol. Cell organelles and nuclei (N) are well-preserved, although some cells contain a few lipid droplets, and electron-lucent cells, indicated by the arrows, are occasionally detected. B: Neurons maintained in 1% O₂ for 24 h and then cultured in 20% O₂ for 3 h with 5 µmol/l amlodipine. Cell organelles and nuclei (N) are almost intact, although lipid droplets (LD) are observed within the cell bodies.
constituents of the membrane in a dose-dependent manner, independent of Ca²⁺ channel modulation. Amlodipine significantly inhibited lipid peroxidation at concentrations as low as 10.0 µmol/l. At a concentration of 10.0 µmol/l, amlodipine inhibited 50% of lipid peroxide formation. We demonstrated that amlodipine clearly inhibited cell death in cultured SHRSP neuron during H/R. Our finding is confirmed by the data previously reported (23). The effective dose, however, was much higher than that reported by in this previous study. This difference in the effective dose may related to differences in the type of cells and the in vitro injury systems employed. The antihypertensive agent amlodipine is a low-clearance, long-acting, vasoselective dihydropyridine calcium antagonist (24, 25). Although calcium antagonists were originally developed for use in the treatment of angina pectoris, they are now used to treat other cardiovascular disorders. Amlodipine lowers intracellular cholesterol levels in smooth muscle cells of human atherosclerotic plaques and prevents cholesterol from accumulating in normal cells from patients with coronary atherosclerosis (9, 26). This suggests that a reduction in the peroxidation and preservation of superoxide dismutase (SOD) may be a common mechanism behind the antiatherosclerotic effects of amlodipine (27). Furthermore, Ito et al. demonstrated that SOD was lower in the hypertrophied myocardia of SHR than in the hearts of age-matched WKY, and superoxide anion generation was much more intense in the myocardia of SHR than those of WKY (28). These reports suggest that SHR have a lower cellular content of antioxidants in the vascular tissue. The antioxidant effects of amlodipine may be attributed to the chain-breaking activity associated with its electron-rich dihydropyridine ring, which is located in the membrane hydrocarbon core, as well as to the effects of the drug on membrane physico-chemical properties, as determined by calorimetric approaches (29). In this study, we also demonstrated that carvedilol (0.1–20.0 µmol/l) protected cortical neurons isolated from SHRSP against H/R-mediated neurotoxicity (Fig. 3). Carvedilol is a vasodilating β-blocker (30) and antioxidant (31) approved for treatment of mild to moderate hypertension. Lysko et al. studied the neuroprotective effects of carvedilol on cultured cerebellar neurons and on CA1 hippocampal neurons of gerbils exposed to brain ischemia (32). Carvedilol protected cultured neurons against glutamate-mediated excitotoxicity (inhibitory concentration [IC₅₀] = 1.1 µmol/l) as well as against a 20 min oxidative challenge (IC₅₀ = 5.0 µmol/l) (32). In this study, we verified that carvedilol and carvedilol consistently prevented cell death in H/R at concentrations of 0.5 to 20.0 µmol/l (Fig. 3). This result is agreement with the data reported previously (32). In this previous study, carvedilol protected cultured neurons in a dose-dependent manner against glutamate-mediated excitotoxicity as well as against a 20 min oxidative challenge. At 10 µmol/l, carvedilol inhibited lipid peroxidation by 50% and 70% in neurons exposed to two different free radical-generating systems (32). Negishi et al. investigated the in vivo production of hydroxyl radicals in the H/R state in SHRSP (6). SHRSP rats are susceptible to neuronal damage as they produce more hydroxyl radicals than WKY. It is suggested that antioxidants effectively capture the hydroxyl radicals produced in the H/R state, thereby preventing neuronal damage. Amlodipine and carvedilol protect neuronal cells from injury induced by oxygen radicals in vitro and from damage due to ischemia and reperfusion in vivo (32). Our studies indicate that amlodipine and carvedilol may be effective for the treatment of cerebrovascular stroke and neurodegenerative diseases associated with excessive apoptosis, although it is not necessarily clear whether amlodipine make the transition to the brain.

Because dipyridamole is a compound with multiple pharmacological actions, we tested whether it was possible to protect SHRSP neurons by mimicking these activities using a combination of other antihypertensives. We have shown that dipyridamole protects neuronal cells of SHRSP from injury induced by H/R. Dipyridamole has been shown to inhibit lipid peroxidation and to scavenge superoxide and hydroxyl radicals (33, 34). In addition, Farinelli et al. demonstrated that dipyridamole was neuroprotective for a variety of rat embryonic central nervous system (CNS) neurons cultured in serum-free basal medium lacking trophic factors or other additives (35). In their study, vitamin E provided neuroprotection, whereas an array of compounds that mimic other actions (inhibition of phosphodiesterases, blockade of nucleoside and chloride transport, interference with the multidrug resistance protein, and enhancement of prostacyclin synthesis) of dipyridamole failed to promote survival (35). These results suggest that a major cause of neuronal death is oxidative stress, which is relieved by dipyridamole. Indeed, administration of insulin, which was not protective alone in a basal medium, along with dipyridamole significantly enhanced long-term neuronal survival (35). Furthermore, in the present study we were unable to confirm the neuroprotective effect of co-treatment with amlodipine and carvedilol. Thus, it was especially important to establish whether the survival-promoting actions of dipyridamole were present in SHRSP neurons during H/R. In contrast, inhibition of cell death in cortical neurons caused by nilvadipine was not confirmed. These results are consistent with the data we reported previously (36). It was demonstrated that nilvadipine is effective against neuronal dysfunction in focal cerebral ischemia (37). Tanaka et al. showed the effect of nilvadipine on the neuronal function of the cat brain after occlusion of the left middle cerebral artery (MCA) for 60 min followed by reperfusion for 90 min (38). Reports suggest that the inhibitory effect of nilvadipine on the neuronal Ca²⁺ influx, in combination with the cerebral vasodilatory action, prevents neuronal damage during brain ischemia (39). Thus, although nilvadipine conferred protection from death, its effects may be caused by inhibition of neuronal Ca²⁺ influx, not antioxidant activity (38). In this study we demonstrated that nilvadipine did not reduce neuronal cell death during H/R. Previously re-
ported data suggest that this agent does not decrease oxygen radicals (38), and this may be the reason why nilvadipine did not prevent cell death.

From these results, amlodipine and carvedilol, with their antioxidant properties, would appear to be useful for the treatment of stroke. Dipyridamole is a compound with multiple pharmacological actions, and it has also been reported to have an antioxidative effect (36, 37). Our results showed that the effects of co-treatment with vitamin E or dipyridamole were not synergistic (Figs. 5, 6). Furthermore, we compared the effects of co-treatment with vitamin E or dipyridamole, vitamin E and dipyridamole, and vitamin E and nilvadipine on ischemic neuronal death in SHRSP and WKY during H/R at the maximally effective doses (Fig. 9). Vitamin E was most effective at a concentration of 100 µg/ml, amlodipine at 5 µmol/l, carvedilol at 10 µmol/l, and dipyridamole at 10 µmol/l (Fig. 9A).

In conclusion, the present findings support the potential use of amlodipine and carvedilol to protect cortical neurons of SHRSP from ischemic cell death. Given the suggested role of free radicals in neuronal death after H/R or trauma (40), in neurodegenerative disorders (41), and in Alzheimer’s disease, which is significantly delayed by vitamin E treatment (42), antihypertensives with their antioxidant properties may have clinical benefits for the prevention of ischemic neuronal death, especially when combined with vitamin E. It is tempting to speculate that agents such as amlodipine and carvedilol would be effective in the treatment of cerebrovascular stroke and neurodegenerative diseases in hypertensive patients.

References

22. Gonzalez-Polo RA, Soler G, Alvarez A, Fabregat I, Fuentes JM: Vitamin E blocks early events induced by 1-methyl-4-


