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Effect of Nilvadipine on the Cerebral Ischemia-Induced Impairment of Spatial Memory and Hippocampal Apoptosis in Rats

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Abstract. We investigated the effects of nilvadipine and amlodipine on the cerebral ischemia-induced impairment of spatial memory in 8-arm radial maze performance and hippocampal CA1 apoptosis in rats. Single cerebral ischemia impaired memory without inducing apoptosis. In these rats, neither nilvadipine nor amlodipine at 3.2 mg/kg, i.p. improved the impaired memory. On the other hand, repeated cerebral ischemia (10 min ischemia × 2, 1 h interval) impaired spatial memory and induced hippocampal apoptosis 7 days after the final occlusion/reperfusion. Moreover, repeated ischemia increased the apoptotic cell number, an effect observed after 3 days and peaked after 7 days. However, mRNA expression of the apoptosis-related early oncogene bax and CPP 32 (caspase-3) was observed after 24 h. In these rats, nilvadipine, but not amlodipine, significantly improved memory, concomitantly decreased hippocampal apoptosis, and suppressed both bax and CPP 32 expression. These results suggest that nilvadipine improved the memory impairment in repeated ischemia by reducing bax and CPP 32 expression and suppressing the induction of apoptosis in the hippocampus. Nilvadipine may have a neuroprotective effect and could be a useful pharmacotherapeutic agent for cerebrovascular dementia.

Keywords: ischemia, spatial memory, nilvadipine, oncogene, apoptosis

Introduction

Voltage-dependent calcium (Ca^{2+}) channel blockers could be effective pharmacotherapeutic agents for some types of brain dysfunction by regulating neuronal Ca^{2+} homeostasis. Their neuroprotective effects in ischemic conditions has been proved experimentally in rats (1, 2) and clinically in the human beings (3). However, the efficacy of voltage-dependent Ca^{2+} channel blockers in this regard depends on whether they are centrally active (nilvadipine) or not (amlodipine).

Nilvadipine is a safe and effective agent prescribed for patients with both hypertension and chronic major cerebral artery occlusion (4). In hypertensive patients with high risk of atherosclerosis, nilvadipine may protect LDL cholesterol from in vivo oxidation (5). Nilvadipine reduces infarction foci (6), improves cerebral microcirculation (7, 8), but does not affect the systemic arterial blood pressure (9). Pathohistochemical studies have revealed that the volume of the infarction in the middle cerebral artery occlusion model could be decreased by nilvadipine (10 – 12).

Although it is a dihydropyridine derivative, nilvadipine has several kinetic, dynamic, and therapeutic effects that are different from even those of the other voltage-dependent Ca^{2+} channel blockers belonging to the same class. Nilvadipine could easily be transmitted to the central nervous system. Moreover, vascular smooth muscles contracted by potassium were reported to be inhibited by nilvadipine (13). It antagonizes both L- and T-type Ca^{2+} channels (14) and exerts an antioxidant effect (15) that is reported to be greater than the effects of nimodipine, nicardipine, and amlodipine (16). It has also been reported that nilvadipine (but

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not amlodipine) increased striatal dopamine and DOPAC contents and protected against motor deficits in mice (17).

Dementia and memory impairment, including the type caused by cerebrovascular disease, are important clinical conditions that are still waiting for successful remedy. Moreover, evaluation of the clinical efficacy of drugs, with known therapeutic value and toxicity, for possible treatment of ischemia and its sequel will be of great interest. In addition, the adoption of a convenient animal model suitable for screening these drugs is also of relevant significance. It has been reported that a single ischemic episode did not induce continuous long-term impairment in spatial memory in an 8-arm radial maze task (18). However, this long-term impairment together with a marked neuronal cell death, especially apoptosis in the hippocampal CA1 field, could be induced when the 10-min ischemic episode was repeated once after an interval of 1 h (19, 20).

The present study aimed at investigating rats' spatial memory impairment induced by single and twice repeated ischemic episodes; the relevant role of the bcl-2 family (bax, bad, bcl-xL), CPP 32 (caspase-3); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in this type of apoptosis. In addion, we compared the effect of nilvadipine and amlodipine on the spatial memory dysfunction.

Materials and Methods

Animals

Male Wistar rats weighing 200 – 250 g (Kyu-Do Co., Ltd., Saga) were housed in groups of 5 per cage in a room with controlled temperature (23 ± 2°C), relative humidity of 60 ± 5%, and 12-h light/dark cycle, light period starting at 7:00 am. Food and water were available ad libitum except the rats used in the 8-arm radial maze task were subjected to a restricted feeding schedule. Animal care and the experimental procedures were based on the regulations of the Animal Care and Use Committee of Fukuoka University.

8-Arm radial maze task

Apparatus: The radial maze apparatus used in this study (Neuroscience Co., Tokyo) was a modified version (21) of that originally adopted by Olton and Samuelson (22). It consisted of equally spaced, transparent plexiglas eight arms (50-cm-long, 10-cm-wide with a transparent 50-cm-high side wall) extending from a central octagonal hub (24-cm-across), surrounded by opaque guillotine doors at the entrance of each arm. The maze was elevated 50 cm from the floor. Food cups (3 cm in diameter, 1 cm in depth, black plexiglas), mounted at the end of each arm, served as receptacles for the reinforcers (2 lumps, 50 – 60 mg crystallized sugar) in the baited arms. The experiments were conducted in a room containing many fixed extra-maze visual cues.

Procedures: *1. Restricted feeding schedule*: The schedule started at the beginning, and continued throughout the experiments. The schedule was achieved by reducing the daily consumption of ration (10 – 12 g /day, CE-2; Clea Japan, Tokyo) so that body weight of each rat was maintained at 80 – 90% of the freely feeding level. Water was always available ad libitum.

2. Pretraining, training, and assessment of maze performance and drug effects: The rats were pretrained in groups (5/group) to the apparatus and the reinforcer food pellets for 3 days, 3 times daily for 10 min repeated after 60 min, before the animals' training. The training phase was started 1 day after the pretraining and was performed 3 times/day for 14 days in order to allow the rats to learn how to perform the maze task. In the training and drug tests trials, each rat was placed in the central platform, then the guillotine was lifted after 1 min, and the rats were allowed to move freely in the maze to the baited arms. The trial continued until the test animal had either entered all 8 arms and consumed the baits or 10 min had elapsed. If the test animals proceeded in the 8-arm radial maze task by using sequential routes consisting of repeating a given angular direction (e.g., 45°) to the neighboring arm, then such animals were excluded from the present experiment. Only the rats that made no errors or only one error for three consecutive days were selected for the study.

Performance assessment: The following parameters were considered the criteria for radial maze performance: 1) the number of correct choices (CC) in the initial 8 chosen arms (entry into an arm that the animal had not previously visited) and 2) number of errors, ES (reentry into an arm that the rat had previously visited). The maze performance was observed by Video Image Motion Analyzer-AXIS 30, Neuroscience Co. (Tokyo).

Induction of four-vessel cerebral ischemia

Four-vessel occlusion was performed according to the method described by Pulsinelli et al. (18) and to our previous study (23). Briefly, the rats were anesthetized with 50 mg/kg, i.p. sodium pentobarbital and immobilized in a stereotaxic apparatus. The bilateral vertebral arteries were electrocauterized with a bipolar coagulator (MICRO-3D; Mizuho Industrial Co., Tokyo). The bilateral common carotid arteries were then exposed and a hydraulic pressure vascular occluder (OC: 1.5-mm diameter; Technical Supply Co., Osaka) was applied to each exposed artery.

In single ischemia, the common carotid arteries were

bilaterally compressed on the next day, with the hydraulic pressure occluders, and cerebral circulation was interrupted for 10 min. In the case of repeated ischemia, the common carotid arteries were bilaterally occluded for 10 min, repeated once after an interval of 1 h. Body temperature was maintained at 37°C using a heating pad and heating lamp until recovery from anesthesia after surgical operation or until the righting reflex reappeared following occlusion of carotid arteries. The rats that did not exhibit loss of their righting reflex during arterial occlusion were excluded from the subsequent experiment. The rats that only underwent cauterization of the vertebral arteries and then were fitted with occluders on the common carotid arteries without occlusion were used as sham-operated controls.

Apoptosis detection

After behavioral testing, the animals were euthanased by deep sodium pentobarbital (50 mg/kg, i.p.) anesthesia and perfused transcardially with heparinized saline followed by 4% paraformaldehyde. The brains were removed, fixed overnight in paraformaldehyde, and stored in phosphate buffer before being cut into 10-μm sections. Each section was stained for apoptotic cells using the terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-deoxyuridinetriphosphate (dUTP) nick-end labeling (TUNEL) technique (TACSII; Trevigen, Gaithersburg, MD, USA). Fluorescein-dUTP-labeled fragmented DNA can be visualized directly by fluorescence microscopy (DMRA; Leica, Cambridge, UK). TUNEL-positive cells (green, FITC) in sections that included the dorsal hippocampus were counted.

RT-PCR of apoptosis-related gene mRNA

At 1, 3, and 7 days following the repeated ischemia, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and decapitated; then the brain was immediately removed into a cryostat, cut into coronal sections, and punched out on an ice-cooled glass stage. Brain tissue was sampled from the dorsal hippocampus at the coordinates 2.8-mm posterior to the bregma, 1.0-mm lateral to the midsagittal line and 3.2-mm ventral to the skull surface according to the atlas of Paxinos and Watson (24). For generating cDNA, the total RNA was isolated from the dorsal hippocampus using RNA isolation reagent, Trizol (Gibco BRL, Grand Island, NY, USA). Then 1 μg of total RNA was reverse transcribed into first strand cDNA using oligo (dT) 12 – 18 primer and Superscript II reverse transcriptase (Gibco BRL). For the polymerase chain reaction (PCR), RT-PCR was performed to assess the expression of bax, bcl-xL, bad, CPP 32, and GAPDH mRNA using PCR reagent system (Gibco BRL, Gaithersburg, MD, USA). PCR reactions were carried out in 50 μL of reaction mixture containing 5 μL 10xPCR buffer, 1 μL 10 mM dNTP mix, 1 μL amplification primer ×2, 1 μL Taq DNA polymerase, 1 μL cDNA, and 40 μL deionized water using a program temperature control system (PC-701; ASTEC, Tokyo). The PCR conditions were 5 min at 94°C, 25 cycles with bax, bad, or GAPDH as standards or 27 cycles with bcl-xL for 1 min at 94°C, 1 min at 58°C, 2 min at 72°C, and finally 7 min at 72°C. Preliminary investigation was conducted according to our previous experiments (25) to determine the relationship between the amount of PCR product and numbers of cycles of amplification. A linearity was obtained at 23 – 30 cycles for GAPDH and other mRNAs. Accordingly, the cycles 25 and 27 were chosen in the present study. The PCR products were quantified using 1% agarose electrophoresis (Gibco BRL, Gaithersburg, MD, USA) with conditioning 20 min, 150 V. The agarose gel was prepared with TBE buffer (Gibco BRL, Gaithersburg, MD, USA). The gel was scanned with ultraviolet irradiation after staining with 1 μg/ml ethidium bromide for 20 min. Finally, the densities of the bands on the agarose gels were measured using the NIH Image program. Semi-quantitative measurements of these apoptosis-promoted genes were expressed as the ratios bax/bcl-xL, bad/bcl-xL, and CPP 32/GAPDH. The oligonucleotide primers, sense and antisense sequences for mRNA of GAPDH, bax, bcl-xL, and CPP 32, were according to the Refs. 25, 26, 27, and 28, respectively. The bad mRNA primers were used with a 147 – 168 base sequence for the sense primer and a 702 – 723 base sequence for the antisense primer according to Genbank, after confirmation with Alw NI restriction enzyme. GAPDH was used as an internal control in RT-PCR and was coamplified with bax, bcl-xL, bad, and CPP 32. In the present study, we detected the change in mRNA of bcl-xL expression instead of bcl-2, because bcl-xL could easily be released from bax/bcl-xL heterodimer and then form bad/bcl-xL heterodimer by itself (29).

Drugs

Amlodipine (Dr. Reddy's Laboratories, Upper Saddle River, NJ, USA) and nilvadipine (Fujisawa Pharmaceutical Co., Ltd., Osaka) were dissolved in 25% polyethylene glycol. The drugs were injected intraperitoneally immediately after each reperfusion following the ischemic episode. An appropriate vehicle for each drug was used as a control. The retention trial was conducted 24 h and 7 days after reperfusion in single and repeated ischemia, respectively.

Statistical analyses

The results were expressed as the mean ± S.E.M. The data for behavioral tests and hippocampal histochemical changes were evaluated using the Kruskal-Wallis test followed by the Mann-Whitney *U*-tests for detecting the significance of differences among the groups. The data for mRNA were analyzed by Student's *t*-test.

Results

Effects of nilvadipine and amlodipine on single ischemia-induced impairment of spatial memory

Single ischemia significantly impaired the retention of spatial memory in the 8-arm radial maze task performed 24 h after reperfusion. Nilvadipine and amlodipine (3.2 mg/kg) did not impair spatial memory in intact rats. Although a slight reduction of the errors was observed in the study (data not shown), the drugs neither significantly increased the correct choices nor reduced the errors done by rats with single ischemia.

Figure 1 shows that repeated ischemia caused a prominent impairment of spatial memory 7 days after reperfusion in rats that received vehicle (CC: 5.3 ± 0.2, ES: 10.6 ± 1). Nilvadipine (3.2 mg/kg, i.p.), when injected immediately after each reperfusion, significantly improved the repeated ischemia-induced impairment of spatial memory 7 days after reperfusion (CC: 6.5 ± 0.2, P<0.05; ES: 3.3 ± 0.7, P<0.01). However, amlodipine (3.2 mg/kg, i.p.) did not improve the impaired memory (CC: 5.8 ± 0.4; ES: 6.6 ± 1.4). The sham-operated rats that were not subjected to ischemia showed almost the same number of CC as non-operated rats in their first 8 choices.

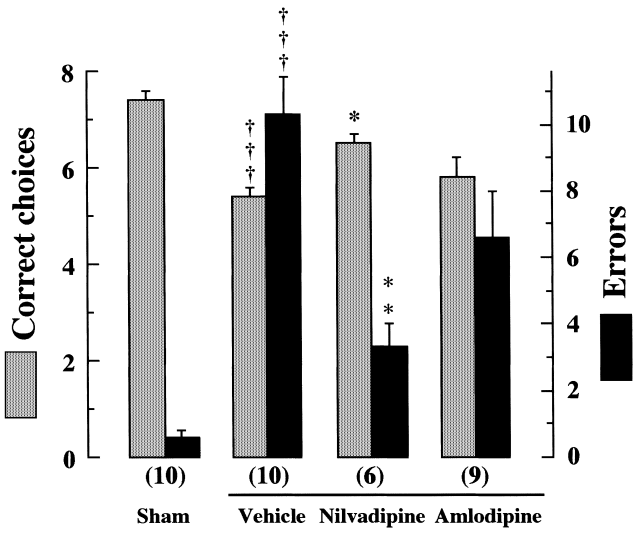

Fig. 1. Effects of nilvadipine and amlodipine (3.2 mg/kg) on repeated cerebral ischemia-induced impairment of spatial memory in the 8-arm radial maze task in rats. The vertebral arteries were cauterized and the common carotid arteries were bilaterally occluded for 10 min, repeated once after an interval of 60 min. Test trial was performed 7 days after the final reperfusion. Each column represents the mean ± S.E.M. The number of rats is given in parentheses at the bottom of each column. †††P<0.001, compared to sham; *P<0.05, **P<0.01, compared to vehicle (Mann-Whitney *U*-test).

Effect of nilvadipine on repeated cerebral ischemia-induced hippocampal apoptosis

The results showed that repeated cerebral ischemia induced hippocampal apoptosis in the CA1 layer. The number of apoptotic cells significantly increased after 3 days and peaked 7 days after the final reperfusion (data not shown). In the light of the behavioral data from the rats with repeated cerebral ischemia, we examined histochemically the effect of nilvadipine on repeated cerebral ischemia-induced apoptosis of CA1 hippocampal cells 7 days after reperfusion. The number of TUNEL-positive apoptotic cells detected in the control, non-ischemic rats that received vehicle was 3.8 ± 0.5, which was 2.1 ± 0.2% of the total 178.8 ± 5.1 cells (Fig. 2: A and D). The number of these cells was significantly (P<0.01, compared to the control) increased in the ischemic rats that received vehicle (66.4 ± 8.6, which was 31.7 ± 3.4% of the total 209.6 ± 6.7 cells; Fig. 2: B and D). Nilvadipine at 3.2 mg/kg, i.p. significantly (P<0.05, compared to the ischemic rats that received vehicle) decreased the number of the TUNEL-positive apoptotic cells (47.9 ± 5.2, which was 23.8 ± 2.3% of the total 201.6 ± 8.1 cells; Fig 2: C and D). Amlodipine at the dose of 3.2 mg/kg, i.p. did not significantly suppress the hippocampal apoptosis (result not shown).

Effect of nilvadipine and amlodipine on the repeated ischemia-induced expression of apoptosis-related oncogenes

Repeated ischemia significantly (P<0.01) increased bax and CPP 32 mRNA expression and ratios of bax /bcl-xL and CPP 32/GAPDH 24 h after reperfusion. (Fig. 3: A and B). These ratios then decreased on day 3 and day 7. On the other hand, the ratio of bad mRNA to bcl-xL mRNA did not significantly change after repeated ischemia, although a non-significant increase was evident on day 3. Figure 3C shows that nilvadipine and amlodipine (3.2 mg/kg, i.p.) significantly (P<0.05) reduced the bax/bcl-xL ratio. However, the results also revealed that only nilvadipine significantly (P<0.01) reduced the CPP 32/GAPDH ratio (Fig. 3D). No effect on bad/bcl-xL was obtained for nilvadipine in this study (data not shown).

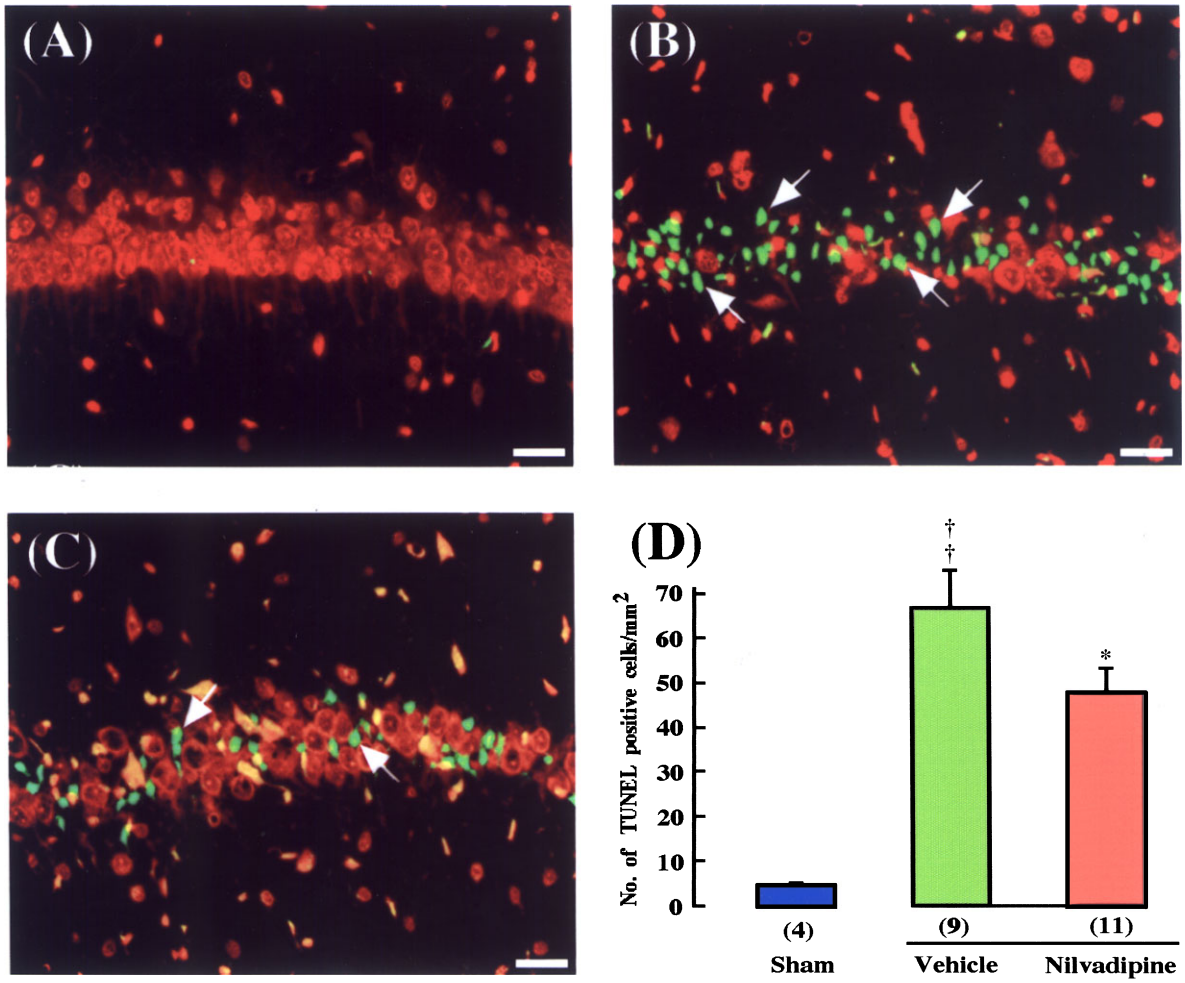

Fig. 2. Histological sections (magnification ×400, calibration bar = 20 μm) of hippocampal CA1 field showing propidium iodide-stained (red) normal and TUNEL-positive FITC-stained (green) apoptotic cells (arrow). A: Sham rats; B: Repeated ischemia + Vehicle; C: Repeated ischemia + Nilvadipine, 3.2 mg/kg; D: The effect of nilvadipine (3.2 mg/kg, i.p.) on rats' hippocampal apoptosis 7 days after induction of repeated cerebral ischemia. Each column represents the mean ± S.E.M. The number of rats is given in parentheses at the bottom of each column. $^{\dagger\dagger}P<0.01$, compared to sham; $^{*}P<0.05$, compared to vehicle (Mann-Whitney *U*-test).

Discussion

In this study, repeated ischemia impaired spatial memory of rats without disturbing the motor function. Repeated ischemia induces central neuronal damages and memory deficit that are different from those induced by single ischemic episodes. In addition to a prominent reduction of acetylcholine release, repeated ischemia induces a greater impairment of spatial memory than the single ischemia (20) and causes hippocampal CA1 cells apoptosis (30). On the other hand, in single ischemia, the spatial memory, disturbed after ischemia-reperfusion, rapidly recovers to the original condition and causes no apoptosis (23, 30). We had previously found that an NMDA-receptor antagonist, MK-801, improved the impairment of spatial memory induced by single cerebral ischemia, but did not improve the impairment or apoptosis induced by repeated cerebral ischemia. We also found that YM-90K, a selective AMPA-receptor antagonist, improved the impairment in spatial memory and apoptosis induced by repeated cerebral ischemia (unpublished data). These results suggest that NMDA receptors could be involved in the impairment of spatial memory induced by single ischemia, whereas AMPA receptor-mediated mechanisms are involved in the impairment caused by repeated cerebral ischemia (19). According to results of this study, it could be suggested that nilvadipine and amlodipine are not active against single ischemia which is accompanied by disturbance of neurotransmitters balance and NMDA

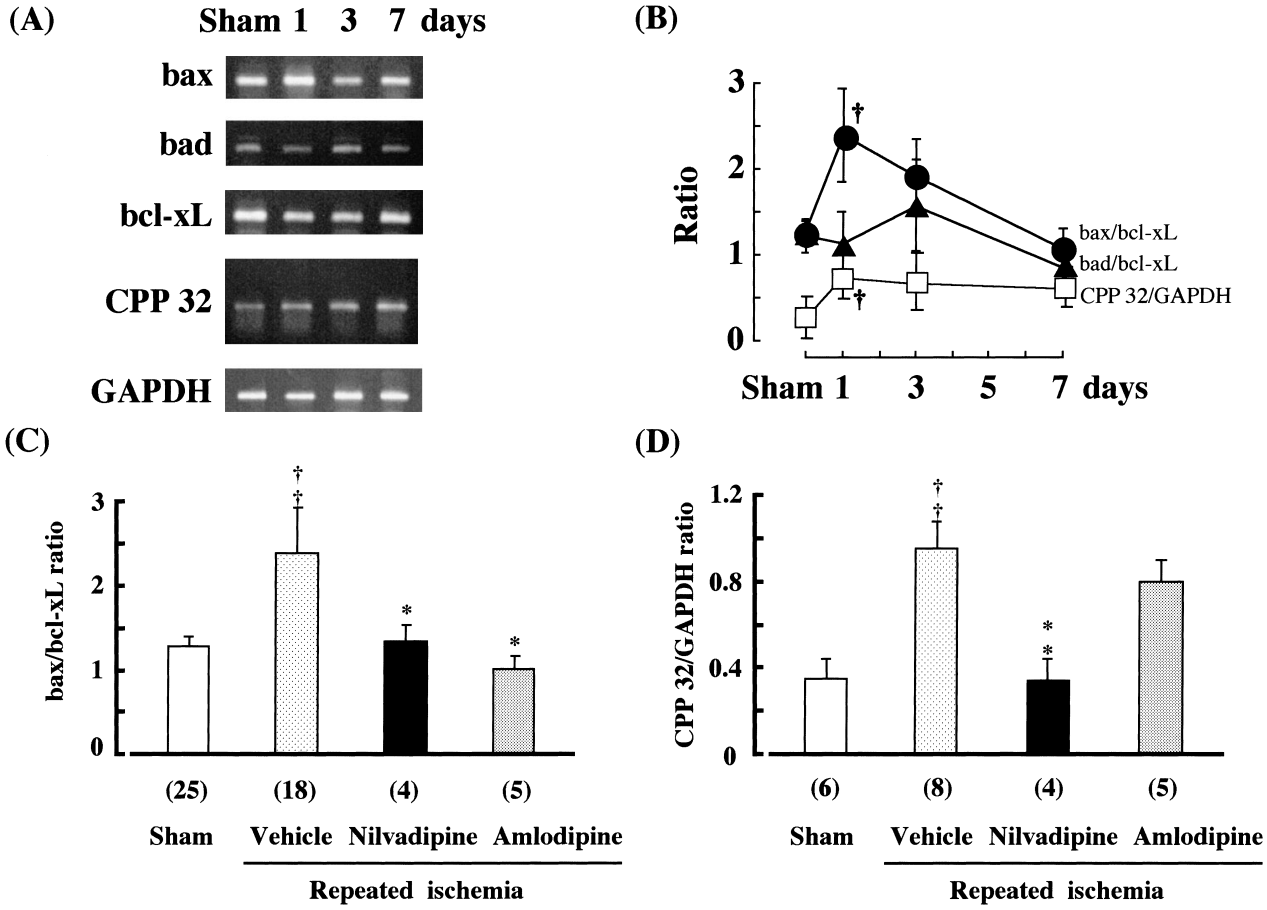

Fig. 3. Effect of repeated ischemia, nilvadipine, and amlodipine on oncogene expression in dorsal hippocampus. A: Densities of bax, bad, bcl-xl, CPP32, and GAPDH mRNA bands on agarose gels stained with ethidium bromide, scaned with UV and measured by NIH Image software. B: Changes in the ratios of mRNA levels for bax/bcl-xL, bad/bcl-xL, and CPP32/GAPDH on days 1, 3, and 7 following repeated ischemia. Data are relative mRNA abundance determined by RT-PCR analysis of extracts from hippocampus and represent ratios of gel bands. $^{\dagger}P<0.05$, compared to sham (Student's *t*-test). C and D: Effects of nilvadipine and amlodipine on ratios of bax/bcl-xL and CPP32/GAPDH, respectively, from hippocampus of rats with repeated cerebral ischemia 7 days after induction of repeated cerebral ischemia. Values are means ± S.E.M. The number of rats is given in parentheses at the bottom of each column in panels C and D. $^{\dagger\dagger}P<0.01$, compared to sham; $*P<0.05$ and $**P<0.01$, compared to vehicle (Student's *t*-test).

receptors activation, but not neuronal cell apoptosis. On the other hand, nilvadipine (but not amlodipine) improves the memory impairment of rats with repeated ischemia that involves apoptosis, with the activation of AMPA receptors in addition to disturbance of the neurotransmitters balance (20, 23).

Ca^{2+} is an important regulator of both cell survival and apoptosis in response to a plethora of pathological insults. Usually, depletion of endoplasmic reticulum and elevation of cytoplasmic and mitocondrial Ca^{2+} are involved in the execution of apoptosis (31). Evidence has been provided that apoptosis induced by ischemia depends on calcium, at least in some cell types. A great deal of interest has been focused on the signaling mechanisms that lead to intracellular Ca^{2+} overload and subsequent apoptosis. One of these mechanisms that are activated in ischemia is glutamate AMPA recepters overactivity. Depolarization of the cell membrane, induced by intracellular Na^{+} influx through AMPA receptors, leads to opening of the L-type Ca^{2+} channel, which is dependent on membrane potential. Subsequently, more Ca^{2+} flows into cells to induce cell death, particularly apoptosis, through the activation of several intracellular enzyme systems including Ca^{2+}-calmodulin-dependent kinase (32 – 34). Nilvadipine, by strongly blocking Ca^{2+} influx through low and high voltage-activated L- or T-type Ca^{2+} channels in the hippocampal CA1 field (8, 35), controls the intracellular Ca^{2+} ion cascade and accordingly could prevent apoptosis and provide protection of neuronal cells in a way

similar to nimodipine (2). However, the effect on Ca^{2+} channels is not the sole mechanism involved in the activity of nilvadipine, because nilvadipine did not improve single ischemia-induced memory impairment that involves extensive Ca^{2+} overload. Moreover, the apoptosis promoter members of the bcl-2 family proteins and activated caspase enzymes, which were over-expressed by repeated ischemia in this study, modulate the intracellular Ca^{2+} dynamics by directly interacting with channels and transporters or forming pores and thus regulate the cellular Ca^{2+} (31). Accordingly, it could be suggested that nilvadipine may affect intracellular Ca^{2+} homeostasis not only by antagonizing Ca^{2+} channels directly, but also via mechanisms mediated by Bax, CPP 32, and GAPDH.

Recently, several oncogenes and intracellular proteases are emerging as triggers of apoptosis. CPP 32 is a cysteine protease implicated in the apoptosis (36, 37). Moreover, recent evidence suggests that GAPDH is a multifunctional protein displaying diverse activities distinct from its conventional metabolic role. GAPDH could act as an early marker of ischemia/reperfusion-induced apoptotic neuronal death (38). The bcl-2-family includes both the apoptosis promoters and suppressers. The promoters include bax and bad, which are mostly in cytoplasm but could be redistributed to the mitochondria during apoptosis (39). The suppressers include bcl-2, (which distributes to several intracellular membranes and may have a major role in control of the permeability of the endoplasmic reticulum), and bcl-xL, which is targeted to the outer mitochondrial membrane (40).

In this study, the repeated ischemia increased CPP 32, bax (but not bad) and bax/bcl-xL, and CPP 32/GAPDH ratio on the first day following ischemia, in a manner similar to that reported previously (30). These results could suggest that disturbance of apoptosis inhibitory and acceleratory gene expression balance toward the apoptosis promoter genes might be important for induction of apoptosis. Moreover, repeated cerebral ischemia induces overexpression of CPP 32 mRNA (37). However, these changes depend on the type of ischemia, brain regions, reperfusion, and time after reperfusion. In this study CPP 32 and CPP 32/GAPDH increased 24 h after reperfusion, but the apoptotic cells started increasing after 3 days and increased more after 7 days. Although the upregulation of mRNA of CPP 32 precedes the activation of CPP 32, its appearance requires a period of time specific to the stroke quiddity: 6 h after middle cerebral artery occlusion (41) and 1 – 3 days (42) or 3 – 7 days after cerebral ischemia (30). It could be suggested that an increase in CPP 32 mRNA expression in the early phase of repeated cerebral ischemia increases CPP 32/GAPDH. The increased CPP 32, by cleaving Poly (ADP-ribose) polymerase, causes nicotinamide adenine dinucleotide depletion and energy failure and eventually apoptotic cell death (43). Moreover, it has been reported that CPP 32 triggers the expression of an apoptosis-enhancing gene, such as bax, which in turn triggers the apoptotic process by translocation from the cytoplasm to the mitochondrial outer membrane, where it competes with and downregulates Bcl-2 (25) and releases cytochrome c to the cytosol, thereby activating the caspase cascade, which in turn cleaves several crucial substrates and initiates the apoptosis (44). However, these changes take place after 3 – 7 days, a gap that may be specific to cerebral ischemia. It should be noted that GAPDH could also participate in this apoptosis-inducing cascade, because overexpression of GAPDH and its nuclear translocation has been reported in both the ischemic core and penumbra area soon after focal ischemia (38). The pattern of reperfusion influences the outcome of repeated ischemia. It has been reported that reperfusion contributes to neuronal damage and DNA ladering which is characteristic of apoptosis occurring earlier in models involving reperfusion (45, 46) than in models of permanent ischemia (47). It could be suggested that the reperfusion in our study (especially the second one) increases neuronal CPP 32 in the CA1 field of the hippocampus, which is particularly sensitive to the ischemic insult, as it was reported in a model of transient global ischemia (37). Moreover, the concomitant increase of CPP 32 and bax are required for induction of apoptosis, but after a lag of 3 – 7 days.

In the present study, nilvadipine suppressed the expression of bax and CPP 32, but did not significantly change the bad, and inhibited apoptosis, suggesting that suppression of bax and CPP 32 together is required for the antiapoptotic and memory improving effect of nilvadipine. The effect of nilvadipine may be associated with binding to GAPDH, as this binding is proposed as a mechanism by which propargylamines (including deprenyl) decrease synthesis of proapoptotic proteins including bax and GAPDH (48), in addition to the central Ca^{2+} antagonistic activity.

It should be noted that amlodipine, also Ca^{2+} L-type channel blocker, reduced the bax/bcl-xL, but not CPP 32/GAPDH, and it neither prevented apoptosis nor improved the deteriorated memory. However, amlodipine has been reported to exert a very potent neuroprotective effect and to inhibit free radical-induced damage to membranous lipid constituents of rat cerebellar granule cells in vitro (49). This result could be due to the poor penetration of amlodipine through the blood-brain barrier. However, it seems that in the ischemic rats, amlodipine could get access to brain due to impairment of the blood-brain barrier. However, the

degree to which amlodipine enters the brain is not sufficient to improve the deteriorated memory. Moreover, these results also indicate that suppressing only bax is not enough to exhibit a memory improving activity in repeated ischemia

In conclusion, the present study demonstrates an improving effect of nilvadipine on the memory impairment induced by repeated ischemia, possibly through prevention of the development of apoptosis. The effect of nilvadipine may involve inhibition of Ca^{2+} influx and decrease of bax/CPP 32 mRNA. These results could suggest that nilvadipine may have a neuroprotective effect and that this drug may be useful for treating cerebrovascular dementia that develops consequent to stroke or other ischemic insults.

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