Iranian Journal of Basic Medical Sciences

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Nimodipine promotes neurite outgrowth and protects against neurotoxicity in PC12 cells

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ARTICLE INFO

Article type:
Original article

Article history:
Received: May 8, 2020
Accepted: Nov 7, 2020

Keywords:
Calcium channels
MAP kinase signaling - system
Neuronal outgrowth
Neuroprotection
Nimodipine

ABSTRACT

Objective(s): Nimodipine is an L-type voltage-dependent calcium channel (VDCC) antagonist. However, the actions of nimodipine except calcium blocking are poorly understood. This study aimed to investigate the effect of nimodipine on neurite outgrowth and neuroprotection *in vitro*.

Materials and Methods: After PC12 cells were treated with different concentrations of nimodipine, neurite outgrowth was estimated using the ImageJ software. Neuroprotective effects of nimodipine against H_2O_2 and calcium ionophore-induced neurotoxicity were investigated using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, the activation of extracellular signal-regulated kinase (ERK) and cyclic AMP-response element-binding protein (CREB) pathway was investigated for clarifying the action mechanism of nimodipine.

Results: Nimodipine treatment at doses of higher than 10 μM induced neurite outgrowth in the cells. Additionally, VDCC knockdown by siRNA significantly suppressed the nimodipine-induced neurite outgrowth in PC12 cells, suggesting that the drug promotes neurite outgrowth by binding to VDCC. H_2O_2 and calcium ionophore induce oxidative and calcium stress in PC12 cells. Nimodipine exhibited neuroprotective effects against H_2O_2- and calcium ionophore-induced neurotoxicity by increasing the mRNA expression levels of neurotrophic factors, calcium-binding proteins, and antioxidants that are transcribed by CREB activation.

Conclusion: This is the first report that nimodipine induces neurite outgrowth and exerts its neuroprotective activity through the ERK/CREB signaling pathway in PC12 cells.

► *Please cite this article as:*
Kusakabe M, Hasegawa Y. Nimodipine promotes neurite outgrowth and protects against neurotoxicity in PC12 cells. Iran J Basic Med Sci 2021; 24:51-57. doi: 10.22038/ijbms.2020.48567.11152

Introduction

Nimodipine, an L-type calcium channel antagonist, produces vasodilation by acting on vascular smooth muscle cells (1, 2). Although the use of nimodipine is restricted mainly to the management of aneurysmal subarachnoid hemorrhage, the drug has been reported to show effects for other uses, such as treatment of dementia associated with cerebrovascular disease and Alzheimer's disease, regeneration of peripheral facial nerve function after maxillofacial surgery, and promotion of functional recovery after intracranial facial nerve crush (3-5). However, it is not clear if these effects are due to the vasodilation caused by nimodipine or to other actions of the drug.

Using both *in vitro* and *in vivo* studies, researchers have shown that the neuroprotective effects of nimodipine occur via its blocking of calcium entry into cells. Nimodipine treatment prevented the death of PC12 cells induced by oxygen–glucose deprivation or *N*-methyl-D-aspartate (6, 7) and protected against alcohol-induced cerebrovascular damage in a rat model of chronic alcoholic encephalopathy (8). The drug also attenuated methylmercury-induced neurotoxicity and behavioral toxicity in mice by inhibiting an overload of the intracellular calcium concentration (9). By contrast, there are some reports about nimodipine acting via mechanisms independent of calcium influx blockage. Nimodipine showed neuroprotective effects against the cytotoxicity induced by alcohol and osmotic stress, as well as against the PC12 cell death induced by nerve growth factor (NGF) deprivation (10, 11). In fact, nimodipine enhanced the NGF-induced neurite outgrowth in PC12 cells without affecting the intracellular calcium concentration (10). *In vivo* studies also showed that co-administration of NGF and nimodipine exerted superior recovery effects in a rat model of sciatic nerve injury regeneration (12) and accelerated axonal regeneration after peripheral nerve injury in rats (13). Nimodipine has also shown activity other than its neuroprotective and neuroregenerative functions, such as stimulation of β-amyloid secretion in neuronal cells and inhibition of lipopolysaccharide-induced microglia activation by a mechanism independent of calcium influx blockage (14, 15). These results suggested that the neuroprotective, neuroregenerative, and other effects of nimodipine do not necessarily rely on its function as a calcium channel blocker. However, the action mechanisms of these other non-calcium-blocking functions of nimodipine are poorly understood. In this study, we investigated the effects of nimodipine on neurite outgrowth in PC12 cells as well as its neuroprotective effects in order to clarify the mechanisms of action of the drug.

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Materials and Methods

Materials

Antibodies against β-actin, extracellular signal-regulated kinase (ERK), T202/Y204-phosphorylated ERK (p-ERK), cAMP-response element binding protein (CREB), S133-phosphorylated CREB (p-CREB), serine/threonine protein kinase (Akt), and S472-phosphorylated Akt (p-Akt) were purchased from Biorbyt (San Francisco, CA, USA). The PC12 cells were purchased from the Japanese Collection of Research Bioresource Cell Bank (Osaka, Japan).

Cell culture and neurite outgrowth

The PC12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% horse serum. For investigating the effects of nimodipine, PC12 cells were seeded at 2 × 10^4 cells/well in 24-well plates coated with Cellmatrix (Nitta Gelatin Inc., Osaka, Japan), and nimodipine was added at the indicated concentrations. After 72 hr, the lengths of the neurites were measured using Image J software in at least 5 randomly selected areas and expressed as the mean±standard deviation (SD).

The toxicity of nimodipine and nifedipine was evaluated with the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (16). In brief, after treatment of the PC12 cells with nimodipine, MTT was added to a final concentration of 0.5 mg/mL in each well. After incubation of the cells for 4 hr at 37 °C, the generated formazan crystals were solubilized in 20% sodium dodecyl sulfate (SDS) and the absorbance of the solution at 570 nm was measured.

The effects of inhibitors on neurite outgrowth were measured as follows. After 48 hr of treatment of PC12 cells with 20 μM nimodipine in the absence or presence of 20 μM of the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 or 10 μM of the protein kinase C (PKC) inhibitor BIM-III, the lengths of the neurites were measured as described above.

For VDCC knockdown experiment, PC12 cells were seeded at 6 × 10^4 cells/well in 6-well plates. After the cells had reached 80% confluency, they were transfected with 0.25 μM of VDCC small interfering RNA (siRNA), using the INTERFERin Kit (Sigma-Aldrich, St. Louis, MO, USA). After 48 hr, the lengths of the neurites were evaluated as described above.

Neuroprotective effect of nimodipine

To investigate the neuroprotective effect of nimodipine against H_2O_2-induced cell death, PC12 cells were seeded at a density of 1 × 10^4 cells/well in 96-well plates, and nimodipine was added at the indicated concentrations. After 2 hr, H_2O_2 was added to a final concentration of 57 mM. After 24 hr, cell viability was determined using the MTT assay.

To investigate the effect of nimodipine on calcium ionophore-induced cell death, PC12 cells were seeded at a density of 1 × 10^4 cells/well in 96-well plates, and nimodipine was added at the indicated concentrations. After 2 hr, calcium ionophore A23187 was added to a final concentration of 0.1 μM. After 24 hr, cell viability was determined using the MTT assay.

Semi-quantitative RT-PCR analysis

PC12 cells were incubated for 48 hr in the absence or presence of nimodipine at the concentrations of 10 and 20 μM. Then, total RNA was extracted from the cells and purified using an RNAiso Plus Kit (Takara, Shiga, Japan) and subjected to the semi-quantitative RT-PCR using primers (Table 1) specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), neurofilament-L, brain-derived neurotrophic factor (BDNF), BDNF receptor (Trk-B), NGF, calretinin, calbindin, calmodulin, heme oxygenase-1 (HO-1), and superoxide dismutase (SOD). The mRNA expression of VDCC after its knockdown by siRNA was also evaluated in the same manner, using specific primers.

The intensities of the amplified bands were estimated using the ImageJ software, where mRNA expression levels were presented as a ratio against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. The amplification cycles used were determined based on the relationship between the amount of PCR product detected and the cycle number.

Western blot analysis

Western blotting was performed as described previously (16). After the cells were collected, sample buffer containing 2% SDS and bromophenol was added,

Table 1. Primer sequences used in semi-quantitative RT-PCR

Gene	Forward primer	Reverse primer
BDNF	5'-TGCTCTTTCTGCTGGAGGAA-3'	5'-GTTGGCCTTTTGATACCGGG-3'
calbindin	5'-ATCCCACCTGCAGTCATCTC-3'	5'-CCCATCATCTCTCTGCCCAT-3'
calcium channel	5'-ATGAGAAGCTGGTGGACTCC-3'	5'-TTTCCATGCTGCCTCCTGTA-3'
calmodulin	5'-TGGGGACTGTGATGAGATCG-3'	5'-GGAAGGCCTCTCGTATCTCC-3'
calretinin	5'-AGGTTCTGGCATGATGTCCA-3'	5'-CGCCAAGCCTCCATAAACTC-3'
GAPDH	5'-TCCTGCACCACCAACTGCTTAG-3'	5'-AGTGGCAGTGATGGCATGGACT-3'
HO-1	5'-CACGCATATACCCGCTACCT-3'	5'-GAAGGCGGTCTTAGCCTCTT-3'
MnSOD	5'-CCTGACCTGCCTTACGACTA-3'	5'-GCTGTGATTGATATGGCCCC-3'
Neurofilament-L	5'-CCCAGCCTACTATACCAGCC-3'	5'-ATCCTTGGCAGCTTCTTCCT-3'
NGF	5'-CCTGAAGCCCACTGGACTAA-3'	5'-GGAGATTGTACCATGGGCCT-3'
TrkB	5'-CCGGCTTAAAGTTTGTGGCT-3'	5'-AGCCACATGATGTCACAGGA-3'

BDNF: brain-derived neurotrophic factor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HO-1: heme oxygenase-1; SOD: superoxide dismutase; NGF: nerve growth factor; TrkB: brain-derived neurotrophic factor receptor

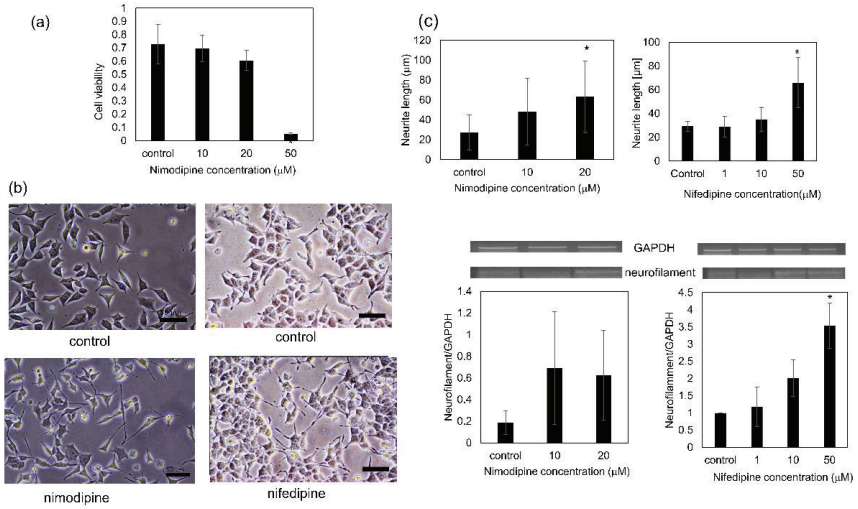

Figure 1. Effect of nimodipine on neurite outgrowth in PC12 cells. (a) Toxicity of nimodipine, as evaluated by MTT assay. (b) Phase-contrast microscopy of PC12 cells in the absence (upper panel) or presence (lower panel) of 20 μM nimodipine (left panel) or 50 μM nifedipine (right panel) for 72 hr. The scale bar represents 50 μm. (c) Estimated length of neurites (n = 100) and level of neurofilament-L expression induced by nimodipine or nifedipine. **P*<0.05 relative to the control
MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

and SDS polyacrylamide gel electrophoresis (17) was carried out. The proteins were electrotransferred onto a polyvinylidene difluoride membrane, which was then incubated at room temperature for 2–6 hr with 5% skim milk (w/v) in a solution containing 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.05% Tween 20 (solution A). The membrane was then reacted overnight with antibodies against CREB, p-CREB, Atk, p-Atk, ERK, and p-ERK. Thereafter, the membrane was treated for 2 hr with an alkaline phosphatase-conjugated secondary antibody, and color development was then carried out using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The band intensities were estimated using the ImageJ software.

Statistical analysis

Each experiment was performed at least twice and usually three times and the data were analyzed using one-way analysis of variance (one-way ANOVA) followed by Turkey's multiple-comparisons test.

Results

Effect of nimodipine on neurite outgrowth

First, we evaluated the toxicity of nimodipine toward PC12 cells (Figure 1a). Nimodipine at a concentration of 20 μM did not induce significant cell death, whereas most cells died at the drug concentration of 50 μM under our experimental conditions. Therefore, 10 and 20 μM of nimodipine were used in the subsequent experiments. On the contrary, nifedipine did not induce cell death even in the concentration of 50 μM (data not shown). Firstly, we evaluated the effects of nimodipine on neurite outgrowth in PC12 cells. Compared with the untreated control cells, the nimodipine-treated cells showed significantly induced neurite outgrowth (Figure 1b), which was distinct under phase-contrast microscopy. The lengths of the neurites from cells treated with 10 and 20 μM nimodipine were approximately 2.0- and 3.0-fold longer, respectively, than those from the control cells. The promotion of neurite outgrowth by nimodipine was also supported by the increase in the mRNA expression of neurofilament-L, which serves as a differentiation biomarker of PC12 cells (Figure 1c). Additionally, we investigated the neurite outgrowth activity in the presence of nifedipine, a dihydropyridine calcium channel antagonist. Nifedipine at a concentration of 50 μM also promoted neurite outgrowth (Figure 1b).

To investigate whether neurite outgrowth occurs via VDCC, knockdown of VDCC 1c subunit was carried out using siRNA. Consequently, the expression of VDCC mRNA was decreased to approximately 50%, and the neurite outgrowth induced by nimodipine was significantly suppressed (Figure 2), suggesting that nimodipine promotes neurite outgrowth by binding to VDCC.

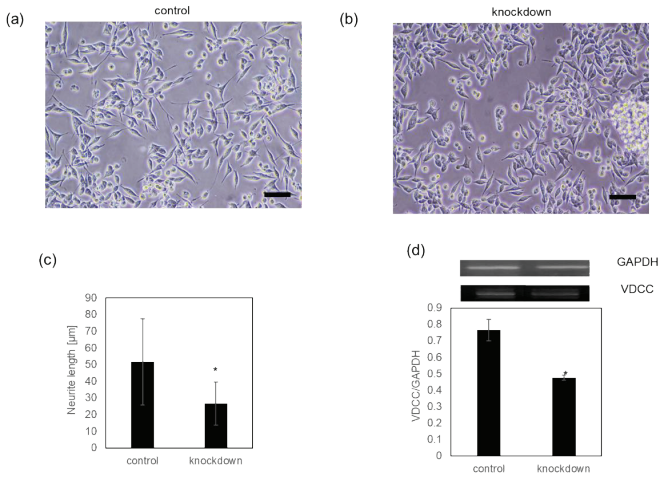

Figure 2. Effect of L-type calcium channel (VDCC) knockdown on nimodipine-induced neurite outgrowth. Phase-contrast microscopy of control cells (a) and knockdown cells (b) after treatment with 20 μM nimodipine for 48 hr. The scale bar represents 50 μm. (c) Length of neurites (n=100) of control cells and knockdown cells after treatment with 20 μM nimodipine for 48 hr was measured in at least 5 randomly selected areas. (d) Level of L-type calcium channel (VDCC) expression, as evaluated by semi-quantitative RT-PCR. **P*<0.05 relative to the control

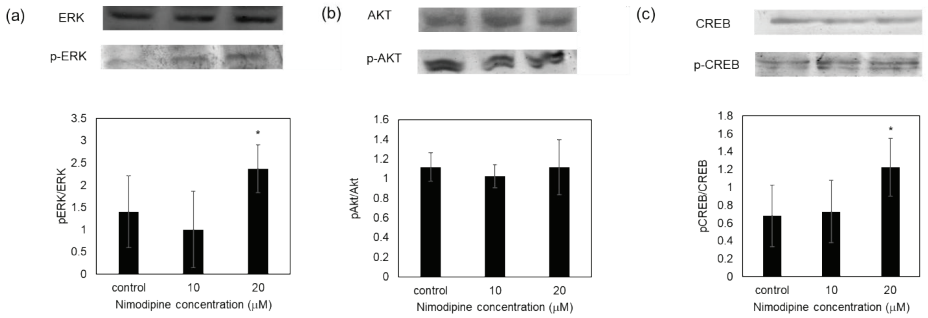

Figure 3. Effect of nimodipine on various signaling pathways. After treatment of the PC12 cells with 20 μM nimodipine for 120 min, the cells were recovered and Western blotting was performed using specific antibodies against (a) ERK and p-ERK, (b) Akt and p-Akt, and (c) CREB and p-CREB. **P*<0.05 relative to the control
ERK: extracellular signal-regulated kinase; CREB: cAMP-response element binding protein; Akt: serine/threonine protein kinase

Intracellular signal transduction by nimodipine

Several studies have suggested that activation of the intracellular signal-transducing proteins (including ERK and Akt) and the transcription factor CREB is involved in neurite outgrowth (18, 19). Therefore, we investigated whether ERK, Akt, and CREB activation is involved in the neurite outgrowth induced by nimodipine. After 120 min of 20 μM nimodipine treatment, the levels of p-ERK and p-CREB, but not p-Akt, were increased significantly in the PC12 cells (Figure 3), suggesting that the drug activates the ERK/CREB pathway.

To verify this result, the effect of an MEK-specific inhibitor, PD98059, which inhibits the phosphorylation of ERK and the PKC inhibitor BIM-III for neurite outgrowth, was investigated (Figure 4). PD98059 and BIM-III did not induce neurotoxicity and neurite outgrowth with the indicated concentration (data not shown). PD98059 significantly suppressed the neurite outgrowth promoted by nimodipine, with the length of the neurites being approximately 20% of that of the control in the presence of 10 μM PD98059. Additionally, the PKC inhibitor BIM-III also suppressed neurite outgrowth, suggesting that the nimodipine promotion of this process is associated with the activation of the PKC/MEK/ERK/CREB pathway.

Expression of neurotrophic factors, calcium-binding proteins, and anti-oxidants

It has been reported that CREB activation induces the mRNA transcription of neurotrophic factors, calcium-binding proteins, and anti-oxidants (20-22). To confirm the activation of CREB by nimodipine, the levels of BDNF, TrkB, NGF, HO-1, and SOD mRNA expression were quantified using semi-quantitative RT-PCR (Figure 5). The treatment of PC12 cells with nimodipine increased the expression of these mRNAs significantly. Moreover, nimodipine increased the expression levels of calcium-binding proteins, which act as calcium buffers in the cells. These results verified that nimodipine can activate CREB in PC12 cells.

Neuroprotective effect of nimodipine

Several studies have reported that CREB activation induces neuronal survival by increasing the expression

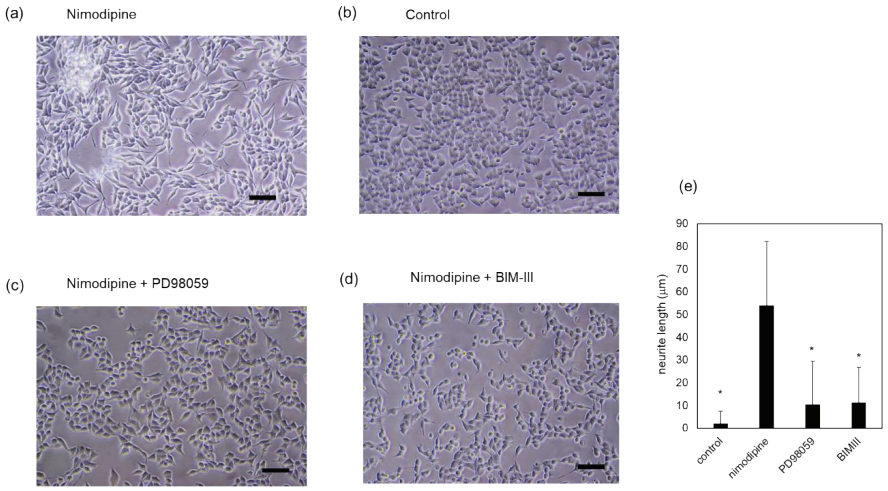

Figure 4. Effects of MEK and PKC inhibitors on nimodipine-induced neurite outgrowth. PC12 cells were treated with 20 μM nimodipine in the absence (a), presence of 10 μM PD98059 (c), or 10 μM BIM-III (d). PC12 cells without nimodipine treatment are shown as control (b). After 72 hr, the lengths of the neurites (n=100) were measured. The scale bar represents 50 μm (e). Bars represent SD. **P*<0.05 relative to the treatment with nimodipine alone
MEK: mitogen-activated protein kinase kinase; PKC: protein kinase C

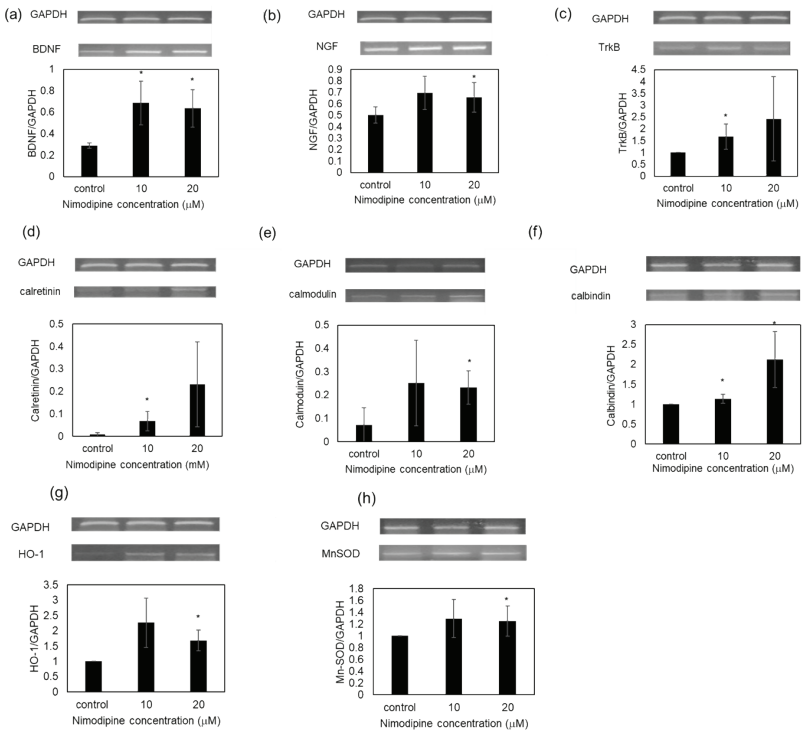

Figure 5. Effect of nimodipine on the expression of neurotrophic factors and their receptor (a–c), calcium-binding proteins (d–f), and antioxidants (g, h). After treatment of the PC12 cells with 20 μM nimodipine for 48 hr, the cells were recovered and semi-quantitative RT-PCR analysis was performed using specific primers. **P*<0.05 relative to the control
GAPDH: glyceraldehyde-3-phosphate dehydrogenase; BDNF: brain-derived neurotrophic factor; NGF: nerve growth factor; TrkB: brain-derived neurotrophic factor receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HO-1: heme oxygenase-1; SOD: superoxide dismutase

of neurotrophic proteins (23, 24). To investigate the neuroprotective effect of nimodipine, its effects against the cytotoxicity induced by H_2O_2 and by the calcium ionophore A23187 were studied (Figure 6). Exposure of the PC12 cells to 72 mM H_2O_2 reduced their viability significantly, resulting in 50% cell death after 24 hr. However, pretreatment of the cells with nimodipine (20 μM) prevented approximately 90% of the H_2O_2-induced cytotoxicity. Treatment of the PC12 cells with A23187 also induced cell death to approximately 40% of the control. In this case, although the protection rendered by nimodipine pretreatment was slight, it was nonetheless statistically significant. These results suggest that nimodipine provides protection against the neuro damage caused by oxidative stress and calcium stress.

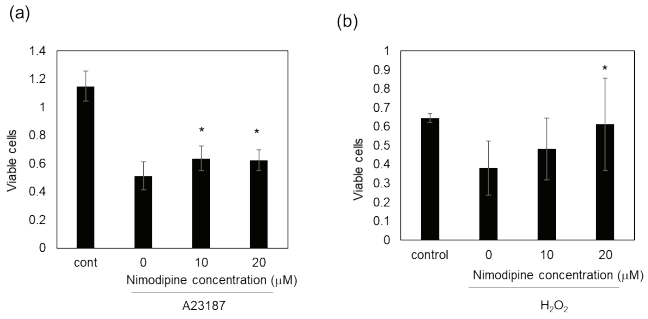

Figure 6. Neuroprotective effect of nimodipine against calcium stress or oxidative stress. (a) PC12 cells were treated with nimodipine at the indicated concentrations, in the absence or presence of 0.1 μM A23187. After 24 hr, the viable cells were estimated by MTT assay. (b) PC12 cells were treated with nimodipine at the indicated concentrations, in the absence or presence of 72 mM H_2O_2. After 24 hr, the viable cells were estimated by MTT assay. **P*<0.05 relative to the treatment with A23187 or H_2O_2 alone
MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Discussion

Using dopaminergic brain slice cocultures, Sygnecka *et al*. (25) showed that nimodipine at the low concentrations of 0.1 and 1 μM could enhance neurite outgrowth. Bork *et al*. (10) showed that nimodipine at 0.1 and 1 μM could increase NGF-induced neurite outgrowth in PC12 cells, but the drug alone could not induce this process. By contrast, our results showed that nimodipine could induce neurite outgrowth at the higher concentrations of 10 and 20 μM. Aside from the difference in the nimodipine concentrations used among these studies, other differences of experimental conditions used, including cell density and incubation time, could have led to the discrepancies in the nimodipine effects on neurite outgrowth in PC12 cells.

In vivo studies have shown that the injection of nimodipine into mice could induce the phosphorylation of TrkB, Akt, ERK, and CREB in the prefrontal cortex and the hippocampus (26). In rats subjected to bilateral occlusion of the common carotid artery, nimodipine administration inhibited memory impairment in the animals through the activation of Akt/CREB in the hippocampus (27). However, it is not clear if

these effects are due to the vasodilatation caused by nimodipine or to direct actions to neuronal cells of the drug. Our results showed that the treatment of PC12 cells with nimodipine induced neurite outgrowth via the PKC/MEK/ERK/CREB signaling pathway. This *in vitro* assay estimating neurite outgrowth will be useful for clarifying the signaling pathway of nimodipine.

Many studies have shown that CREB activation promotes neurite outgrowth and triggers the expressions of neuroprotective and anti-oxidative proteins, including Bcl-2, BDNF, and HO-1, thereby promoting neuronal survival (28, 29). Our results showed that nimodipine treatment of PC12 cells increased the mRNA expression levels of NGF, SOD, calbindin, calmodulin, calretinin, HO1, and BDNF that are transcribed by CREB activation (30), verifying that the drug can activate CREB and exerts neuroprotective effects against oxidative stress and calcium stress in PC12 cells. These results are in accord with those of *in vivo* studies showing that nimodipine administration up-regulated Ca^{2+}-binding proteins in cortical neurons (7, 31, 32) and exerted neuroprotective effects by suppressing oxidative stress in young rats with pilocarpine-induced seizures (33).

Li *et al*. (15) showed that nimodipine, but not nifedipine, significantly attenuated the lipopolysaccharide-stimulated production of nitric oxide, tumor necrosis factor-alpha, interleukin-1beta, and prostaglandin E2 in the microglia. However, the effect was observed at high nimodipine concentrations of 10 and 30 μM (15). Moreover, the binding of [^{3}H]-nimodipine to cardiac, smooth, and skeletal muscle and brain membrane was shown to be specific, with an affinity constant of 0.27 nM (34, 35). These studies suggested that nimodipine may exert its effects via a mechanism other than its binding to VDCC. By contrast, our results showed that neurite outgrowth was decreased by the siRNA-mediated knockdown of VDCC and that it was also promoted by the dihydropyridine calcium channel antagonist nifedipine, suggesting that the binding of nimodipine to VDCC activates CREB and promotes neurite outgrowth. The discrepancy in the results obtained by the different studies may reflect differences in the actions of nimodipine toward the inflammatory response in microglia and neurite outgrowth in PC12 cells. Further studies will be needed to clarify the signaling pathway affected by nimodipine. Despite that we still do not know how nimodipine activates ERK after binding to VDCC, our *in vitro* assay system with PC12 cells together with the use of siRNA or various inhibitors will be useful for clarifying the signal transduction pathways and mechanisms of action of this drug.

Conclusion

Our results show that nimodipine exerts its neurite outgrowth promotion and neuroprotective activities through the ERK/CREB signaling pathway in PC12 cells, and indicate that the neuroprotective effect occurs through a mechanism other than the blockade of calcium entry into the cells. These neuroprotective and neuroregenerative functions of nimodipine give it potential to serve as a therapeutic drug for neurodegenerative diseases.

Acknowledgment

This work was partially supported by the Japan Society for the Promotion of Science KAKENHI (grant No. 16K07865). The results presented in this paper were part of a master's degree thesis by Mrs Miduki Kusakabe.

Conflicts of Interest

The authors declare no conflicts of interest.

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