

## Nicotinic Enhancement of Proliferation in Bovine and Porcine Cerebral Microvascular Endothelial Cells

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Nicotinic acetylcholine receptors are found in microvascular endothelial cells. To reveal the functional role in cerebral angiogenic processes, we studied the nicotinic modulation of proliferation activity in cultured bovine and porcine cerebral microvascular endothelial cells. The proliferation activity was determined by an increase in the number of cells present in culture dishes. When the bovine cerebral endothelial cells at different passages were cultured in the presence of nicotine (10 nM), the proliferation activities were significantly increased in the cells at passage 1 and passage 3, but not at passage 4. Reverse transcriptase-polymerase chain reaction studies demonstrated that the expression of mRNAs coding for  $\alpha 3$  nicotinic receptor subunit was significantly reduced in the bovine cerebral endothelial cells at passage 4, compared with that at passage 1. The proliferation of porcine cerebral endothelial cells (passage 1) was enhanced by acetylcholine (10 nM—100  $\mu$ M) in the presence of atropine, a muscarinic antagonist, and this enhancing effect was inhibited by hexamethonium (100  $\mu$ M, a nicotinic antagonist). The stimulation by acetylcholine (1  $\mu$ M, with atropine) or nicotine (10 nM) induced the phosphorylation of a mitogen-activated protein (MAP) kinase (extracellular-signal regulated kinase: ERK) in the serum-starved endothelial cells. In the presence of PD98059 (2  $\mu$ M, a MAP kinase kinase inhibitor) and atropine, acetylcholine (1  $\mu$ M) failed to enhance the proliferation of porcine cerebral endothelial cells. These results demonstrate that nicotinic stimulation promotes the proliferation of bovine and porcine cerebral microvascular endothelial cells, at least in part, through the MAP kinase activation.

**Key words** nicotinic receptor; cell growth; mitogen-activated protein (MAP) kinase; brain capillary

Neuronal nicotinic acetylcholine receptors are widely distributed in the brain.<sup>1)</sup> They are pentameric ligand-gated ion channels made up of a combination of twelve different subunits ( $\alpha 2$ — $\alpha 10$ ,  $\beta 2$ — $\beta 4$ ).<sup>2,3)</sup> The nicotinic receptors are involved in cognitive processes such as learning and memory in the cerebral cortex and hippocampus.<sup>4)</sup>

Recently, the neuronal nicotinic receptors have been found in many non-neuronal cells, including peripheral and cerebral vascular endothelial cells.<sup>5–7)</sup> It has been shown that nicotine stimulates DNA synthesis, proliferation and tube formation through activation of nicotinic receptors in bovine pulmonary artery endothelial cells, human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells.<sup>8–10)</sup> These observations indicate that nicotinic receptors in the peripheral endothelial cells can contribute to angiogenesis in which new blood vessels are formed from pre-existing vasculatures. It is well known that the endothelial cells display remarkable heterogeneity in different organs.<sup>11,12)</sup> Therefore, an important and incompletely solved question is whether nicotinic receptors play a role in the cerebral angiogenesis, as well as the promotion of angiogenic processes in the peripheral vascular endothelial cells.

The cerebral angiogenesis occurs in hypoxic and/or ischemic conditions, particularly in infarctions and infectious processes, to ameliorate the already disturbed brain conditions, whereas the angiogenesis exacerbates malignant glioma *via* increase in blood supply.<sup>13)</sup> Because the endothelial cell proliferation is a crucial step of angiogenesis, we investigated whether nicotinic stimulation promotes the proliferation of bovine and porcine cerebral microvascular endothelial cells.

### MATERIALS AND METHODS

**Materials** All drugs used were purchased from Sigma (St. Louis, MO, U.S.A.), unless indicated. Hexamethonium was purchased from Wako Pure Chemicals (Osaka, Japan). A rabbit anti-p44/p42 mitogen-activated protein (MAP) kinase antibody and a rabbit anti-phospho-p44/p42 MAP kinase antibody were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.).

**Isolation of Brain Microvessels** We adopted the previously described procedures<sup>14)</sup> for isolation of bovine brain microvessel endothelial cells (BBMEC) and porcine brain microvessel endothelial cells (PBMEC). In brief, cerebrocortical regions were isolated from bovine and porcine brains, and gray matter was collected and homogenized in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Microvessel fragments were trapped on 150- $\mu$ M nylon meshes, and were digested in collagenase type III (Gibco BRL, Life Technologies, Rockville, MD, U.S.A.), trypsin (Worthington Biochemical, Lakewood, NJ, U.S.A.) and DNase I (Worthington Biochemical) in 10% FBS-containing DMEM for 1 h at 37°C. After removal of debris using 200- $\mu$ M nylon mesh, the brain microvessel fractions were re-suspended in FBS with 10% dimethyl sulfoxide, and stored in liquid nitrogen until use.

**Culture of Endothelial Cells** Bovine and porcine cerebrocortical microvessels were seeded in plastic flasks coated with collagen type IV and fibronectin (Roche, Mannheim, Germany) and allowed to adhere for 5 h in 10% FBS-containing DMEM. The medium was then replaced with a growth medium (GM+) consisting of a 1:1 mixture of 10% FBS-containing DMEM and rat astrocyte-conditioned

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medium supplemented with heparin (100 U/ml). The astrocyte-conditioned medium was prepared as mentioned by Gaillard *et al.*<sup>14</sup>) During the preparation, the astrocytes were not stimulated with nicotinic reagents in the present study. The outgrowing cells from brain microvessel fragments, mostly endothelial cells, were cultured at 37°C in a 10% CO<sub>2</sub> atmosphere. At nearly 70% confluence (day 4 or 5 after seeding of microvessels), the primary cultured endothelial cells were harvested with trypsin-EDTA solution (Sigma, T4299, 5 U/ml) for 2 min. All of the obtained cells were endothelial cells (passage 1) as assessed by uptake of acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL, Biomedical technologies, Stoughton, MA, U.S.A.) and by positive staining for von Willebrand factor (data not shown).

All procedures shown above were approved by the Toyama Medical and Pharmaceutical University Animal Research Committee.

**Proliferation Assay** BBMEC and PBMEC were plated at  $6 \times 10^3$  cells and  $2 \times 10^4$  cells/35 mm-diameter dish coated with collagen type IV, respectively. After incubation at 37°C for 2 h to allow the cells to adhere, the cells were treated with growth medium (10% FBS-containing GM+) containing either nicotine or acetylcholine. For experiments to study the influence of a nicotinic antagonist and a MAP kinase kinase inhibitor on the acetylcholine actions, these drugs were applied with atropine 20 min and 2 h before application of acetylcholine, respectively. The medium was exchanged and refreshed at day 2, 5, 8, 11 and 14 (if any). For counting the cell numbers, all the cells were detached from dishes by treating with endothelial-cell trypsin. Trypan blue was added into the cell suspension to exclude dead cells, and the number of living cells was counted using the image analysis software (Win Roof, Mitani, Fukui, Japan).

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)** Total RNA was extracted from proliferating BBMEC (approximately  $1 \times 10^5$  cells/35 mm-diameter dish) using the RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.). The primers listed on Table 1 were used, as described previously.<sup>15,16</sup> These sets of primers were located on different exons to rule out genomic DNA amplification. RT-PCR was performed using 0.3  $\mu$ g total RNA and SuperScript One-Step RT-PCR kit (Life Technologies, Grand Island, NY, U.S.A.) according to the manufacturer's instructions: The RT reaction was performed at 50°C for 30 min, followed by a denaturation step at 94°C for 2 min. Subsequently, 35 and 40 cycles (94°C, 30 s; 58°C, 45 s; 72°C, 45 s) of PCR were run to obtain the PCR products of  $\beta$ -actin and nicotinic receptor subunits, respectively. The numbers of PCR cycles chosen were within the linear range of the amplification: the numbers of PCR-cycles that were tested for linearity were 33–37 and 35–40, respectively before settling on the values given. PCR products were visualized by ethidium bromide staining of agarose gels. Densitometric analyses were performed on Fluor-S Multi-Imager (Bio-Rad, Hercules, CA, U.S.A.). The ratios of fluorescence intensity from bands of bovine  $\alpha 3$  transcripts to that of  $\beta$ -actin transcript were calculated to determine the relative expression level of each subunit mRNA. PCR products were identified by sequencing (data not shown). In fact, RT-PCR performed in the absence of reverse transcriptase did not yield fragments of the expected size

Table 1. PCR Primer Sequences

Subunit	Primer sequences (5'—3')	Product size, bp
$\alpha 3$	For: CGGTCATCATCCAGTTTGAGGT Rev: TCCTGTAGTGCCAGTTGAGCA	815
$\alpha 5$	For: GTTCATGTACCATCGACGTCA Rev: TTCCAATCTTCAACAAGCTC	759
$\alpha 7$	For: GAGTGCTGCAAAGAACCCTAC Rev: GGAACCAGGCACACCAGTTCA	397
$\beta 4$	For: TGAACCTCGCTTCGGCTAC Rev: CATAACTGGGGTCTGGTGG	706
$\beta$ -Actin	For: ATCATGTTTGAGACCTTCAACACCCAGCC Rev: AAGAGAGCTCGGGGCATCGGAACCGTCA	422

For, forward; Rev., reverse.

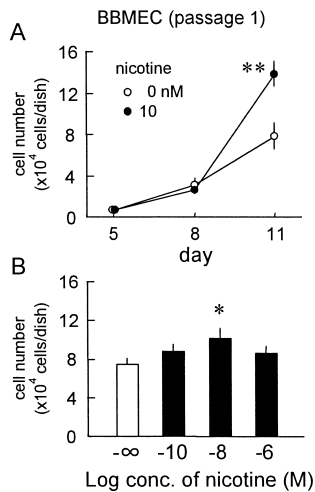
(data not shown).

**Western Blotting** Phosphorylation of p42 MAP kinase (extracellular-signal regulated kinase 2: ERK2) was analyzed by Western blotting. BBMEC and PBMEC were starved for 48 h in serum-free DMEM at 60–70% confluence. The cells were treated with nicotine for 15 and 30 min or bFGF for 10 min at 37°C. When nicotinic stimulation was conducted by combined application of atropine+acetylcholine, the serum-starved cells were treated with atropine (1  $\mu$ M) for 20 min, and then stimulated by acetylcholine for 15–30 min. Subsequently, the cells were solubilized and centrifuged. Proteins in the supernatant were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. The membranes were treated with specified antibodies, and then incubated with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit immunoglobulin, Amersham-Pharmacia Biotech, Piscataway, NJ, U.S.A.). Blots were detected using ECL enhanced chemiluminescence kit (Amersham). The density of protein bands was quantified with Fluor-S Multi-Imager (Bio-Rad, Hercules, CA, U.S.A.). Relative phosphorylation level of ERK2 was represented as the ratio of the value obtained for the phosphorylated ERK2 to that for the total ERK2 in each sample.

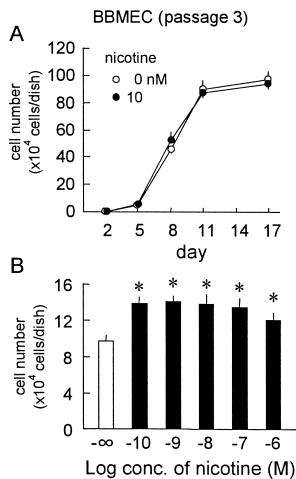
**Statistical Analysis** The significance of differences between two groups was assessed by Student's *t*-test, and the difference between multiple groups was assessed by one-way analysis of variance (ANOVA) followed by the Scheffè's multiple range tests. Values of *p* less than 0.05 were considered to be significant.

## RESULTS

**Enhancing Effect of Nicotine on the Proliferation of BBMEC at Passage 1** To investigate whether the proliferation of cerebral microvascular endothelial cells is modulated by nicotinic stimulation, BBMEC (passage 1) were incubated with nicotine and subjected to cell counts at day 5, 8 and 11 in culture. The proliferation activity of BBMEC at passage 1 was enhanced in the presence of nicotine (10 nM), as determined by an increase in the number of cells present in culture dishes at day 11 (Fig. 1A). The number of cells was 1.8-fold increased by nicotine, compared with control without nicotine. In addition, nicotine (0.1, 10 nM) concentration-dependently enhanced the cell proliferation at day 11 (Fig. 1B). Interestingly, nicotine at a higher concentration (1  $\mu$ M) pro-



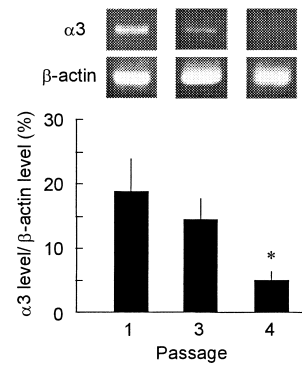
**Fig. 1. Nicotine Enhances Proliferation of Cultured BBMEC at Passage 1**  
 The number of cells was counted to address the activity of cell proliferation. (A) Time-dependent changes in the cell numbers in the presence (●, 10 nM) and absence (○) of nicotine. (B) Effect of nicotine on the BBMEC proliferation observed at day 11. All values are mean ± S.E., n = 6 per group. \* p < 0.05, \*\* p < 0.01 compared with control response without nicotine.



**Fig. 2. Effect of Nicotine on the Proliferation of BBMEC at Passage 3**  
 The numbers of cells was counted to address the activity of cell proliferation. (A) Time-dependent changes in the cell numbers in the presence (●, 10 nM) and absence (○) of nicotine. The data values are mean ± S.E., n = 3–6 per group. (B) Effect of nicotine on the BBMEC proliferation observed at day 6. The values are mean ± S.E., n = 7 per group. \* p < 0.05 compared with control response without nicotine.

duced less potent effect rather than the response to nicotine at 10 nM.

**Enhancing Effect of Nicotine on the Proliferation of BBMEC at Passage 3** In BBMEC that were passaged 3 times, the basal proliferation activity was dramatically increased, and the cells grew up to nearly confluent level within 11 d (Fig. 2A). The number of BBMEC in the confluent state (at day 17) was not affected by treating with nicotine (10 nM). The cell numbers at day 8 tended to be increased by nicotine (10 nM), although the difference did not reach the significant level. Since it may be possible that nicotine only affects the earlier phase of proliferation, the effect of nicotine was precisely examined at day 6 in culture where the cell densities (nearly 1 × 10<sup>5</sup> cells/35 mm-diameter dish) were similar to those of BBMEC (passage 1) at day 11, as shown above (Fig. 1B). In fact, the proliferation of BBMEC



**Fig. 3. Expression of α3 Nicotinic Receptor Subunit in Cultured BBMEC, Determined by RT-PCR**

Upper. Photo-images demonstrating the RT-PCR product for α3 mRNA that yielded the expected band of 815 bp, and the product for β-actin mRNA that showed the expected band of 432 bp in BBMEC at passage 1, 3 and 4. Lower. Semi-quantified expression levels of the α3 mRNA in BBMEC at passage 1, 3 and 4. The level of α3 PCR product was normalized as a percentage of the level of β-actin PCR product in each sample, which was considered 100%. Data values are mean ± S.E., n = 8–9 per group. \* p < 0.05 compared with the normalized expression level in BBMEC (passage 1).

(passage 3) was significantly enhanced in response to nicotine (0.1 nM–1 μM) (Fig. 2B). The maximal response was obtained at 1 nM and corresponded to a 1.4-fold increase in the cell numbers compared with control without nicotine, although less potent effect was produced at a higher concentration (1 μM).

**No Effect of Nicotine on the Proliferation of BBMEC at Passage 4** In the BBMEC that were passaged 4 times, the basal proliferation activity was equally elevated, compared with that of BBMEC at passage 3. However, the proliferation of BBMEC (passage 4) was not affected by nicotine (0.1, 1, 10, 100, 1000 nM): for instance, at day 6 in culture, the cell numbers in the presence of nicotine (1, 10 nM) were 8.0 × 10<sup>4</sup> ± 0.5 × 10<sup>4</sup> cells/dish (n = 5) and 7.3 × 10<sup>4</sup> ± 0.5 × 10<sup>4</sup> cells/dish (n = 6), respectively, which were not significantly different from those in control response without nicotine (7.3 × 10<sup>4</sup> ± 0.3 × 10<sup>4</sup> cells/dish, n = 7). In addition, we reduced the FBS concentration in the growth medium from 10 to 5% in order to avoid the saturation of basal proliferation activity. Nevertheless, nicotine (10 nM) had no effect on the proliferation of BBMEC (passage 4) throughout the observation period from day 5 to day 14 (data not shown).

**Expression of Nicotinic Receptors in BBMEC at Different Passages** Expression of mRNAs coding for α3, α5, α7 and β4 nicotinic receptor subunits in BBMEC was analyzed by a RT-PCR assay. The α3 transcript was detected in BBMEC at passage 1 (Fig. 3). However, the expression level of α3 transcript was not maintained after several passages: relative expression levels of the α3 transcript to the β-actin transcript tended to decrease at passage 3, and they decreased significantly at passage 4 when compared with those at passage 1. The transcripts of α5, α7 and β4 subunits were not detected under the present condition (data not shown).

**Influence of Nicotinic Stimulation on the PBMEC Proliferation** Several drugs are known to exhibit different sensitivities between bovine and porcine cultured endothelial cells.<sup>17)</sup> Therefore, we further investigate the influence of nicotinic stimulation on the proliferation of PBMEC at passage 1. When the PBMEC were treated with nicotine (0.1, 10, 1000 nM), significant enhancement of the cell prolifera-

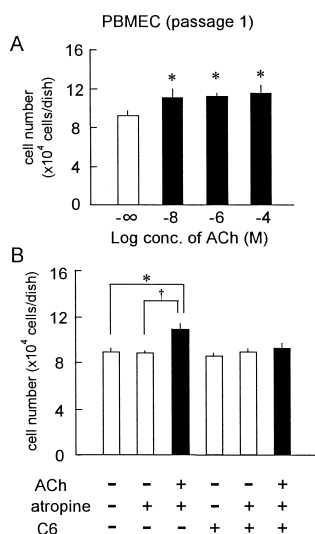


Fig. 4. Acetylcholine Enhances the Proliferation of Cultured PBMEC through the Nicotinic Receptor Activation

The number of cells was counted to address the activity of cell proliferation at passage 1. (A) Effect of acetylcholine (ACh) on the PBMEC proliferation observed at day 8. Atropine ( $0.5 \mu\text{M}$ ) was added to the medium to block muscarinic acetylcholine receptors. The data values are mean  $\pm$  S.E.,  $n=8-9$  per group. \* $p<0.05$  compared with control response without ACh. (B) Effect of hexamethonium (C6,  $100 \mu\text{M}$ ), a nicotinic antagonist, on the proliferation of PBMEC in the presence (black column) and absence (open column) of acetylcholine ( $1 \mu\text{M}$ ) associated with a muscarinic antagonist atropine ( $1 \mu\text{M}$ ) were examined at day 8 in culture. The values are mean  $\pm$  S.E.,  $n=8$  per group. \* $p<0.05$  determined by one-way ANOVA followed by Scheffé's tests. † $p<0.05$  determined by *t*-tests.

tion by nicotine was observed in only 2 out of 10 independent experiments (data not shown). Next we examined the effect of acetylcholine on the PBMEC (passage 1) proliferation at day 8 in culture where the cell densities (nearly  $1 \times 10^5$  cells/35 mm-diameter dish) were similar to those of BBMEC used above (Figs. 1B, 2B). Before the application of acetylcholine, atropine was added to the culture medium to block muscarinic acetylcholine receptors<sup>18</sup>. In fact, atropine ( $0.5 \mu\text{M}$ ) alone did not affect the cell proliferation: the cell numbers in the presence of atropine ( $0.5 \mu\text{M}$ ) were  $8.6 \times 10^4 \pm 0.3 \times 10^4$  cells/dish ( $n=4$ ), whereas the cell numbers in control response without the drug were  $8.9 \times 10^4 \pm 0.4 \times 10^4$  cells/dish ( $n=8$ ). When acetylcholine ( $10 \text{ nM}$ – $100 \mu\text{M}$ ) was applied in the presence of atropine ( $0.5 \mu\text{M}$ ), the cell numbers were significantly increased, compared with the control (Fig. 4A).

#### Blocking Effect of Nicotinic Antagonist on the Acetylcholine-Elicited Enhancement of PBMEC Proliferation

To investigate whether acetylcholine was acting through a nicotinic receptor to enhance the PBMEC proliferation, we examined the effect of hexamethonium, a ganglionic nicotinic receptor antagonist to which  $\alpha 7$  nicotinic receptor displays a poor sensitivity.<sup>19,20</sup> The cell numbers in control response without drugs at day 8 in culture did not differ from those in the presence of atropine ( $1 \mu\text{M}$ ) alone, hexamethonium ( $100 \mu\text{M}$ ) alone, or hexamethonium ( $100 \mu\text{M}$ ) plus atropine ( $1 \mu\text{M}$ ) (Fig. 4B). Stimulation by acetylcholine ( $1 \mu\text{M}$ ) caused the significant increase in the number of PBMEC in the presence of atropine ( $1 \mu\text{M}$ ), but this stimulatory effect was not observed in the presence of hexamethonium ( $100 \mu\text{M}$ ).

#### Involvement of MAP Kinase in the Nicotinic Enhancement of Proliferation in Bovine and Porcine Cerebral Mi-

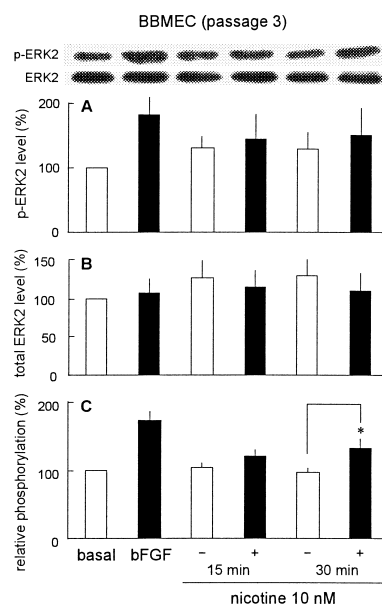


Fig. 5. Nicotine-Induced Phosphorylation of ERK2 in BBMEC

Serum-starved BBMEC (passage 3) were treated with either bFGF ( $30 \text{ ng/ml}$ ) for 10 min or nicotine ( $10 \text{ nM}$ ) for the indicated times, and then lysed for Western blotting analysis. Blots shown are representative of four independent Western blotting analysis demonstrating the effect of nicotine ( $10 \text{ nM}$ ) on the phosphorylated ERK2 (p-ERK2) level and the total ERK2 expression level. Graphs show p-ERK2 level (A), total ERK2 level (B), and relative phosphorylation level of ERK2 in BBMEC (C). The relative phosphorylation level was calculated as the ratio of the density of the p-ERK2 band to that of the total ERK2 band in each sample. Data were expressed as percentages of the basal level (basal) obtained before stimulating by bFGF or nicotine in each experiment. Then, the results were averaged and presented as means  $\pm$  S.E. ( $n=4$ ). \* $p<0.05$  compared with the levels in the non-stimulated cells at each time-point.

**crovascular Endothelial Cells** To clarify the signal transduction mechanism involved in the enhancement, we investigated phosphorylation level of p42 MAP kinase (ERK2) in BBMEC (passage 3) following nicotine stimulation. BBMEC were starved for 48 h at 60–70% confluence, and then we measured the basal level of phosphorylated ERK2 (Fig. 5A) and total ERK2 (Fig. 5B) to calculate the relative phosphorylation level of ERK2 (Fig. 5C). In a positive control experiment, bFGF was added to the culture medium. Ten minutes after the treatment with bFGF ( $30 \text{ ng/ml}$ ), the relative phosphorylation level of ERK2 was 1.7-fold increased, compared with the basal phosphorylation level obtained before the bFGF treatment. When BBMEC were treated with  $10 \text{ nM}$  nicotine for 30 min, the relative phosphorylation level was significantly (1.4-fold) higher than that in non-stimulated cells at each time-point (Fig. 5C). In any cases, no significant changes in the total ERK2 level were observed (Fig. 5B). In the presence of a low concentration ( $2 \mu\text{M}$ ) of PD98059, an inhibitor of MAP kinase kinase (MEK, an upstream regulator of ERK), the relative phosphorylation level of ERK2 was not changed by the treatment with  $10 \text{ nM}$  nicotine (data not shown).

We also investigated the influence of nicotinic stimulation on the ERK2 phosphorylation in PBMEC (passage 1) (Fig. 6). The cells were starved for 48 h at 60–70% confluence, and then treated with atropine ( $1 \mu\text{M}$ ) for 20 min. In a positive control experiment, bFGF ( $30 \text{ ng/ml}$ , 10 min) caused a 2.4-fold increase in the relative phosphorylation level of ERK2 (Fig. 6C). When acetylcholine was added to the medium containing atropine ( $1 \mu\text{M}$ ), the phosphorylation level of ERK2 was progressively increased: at 15 and 30

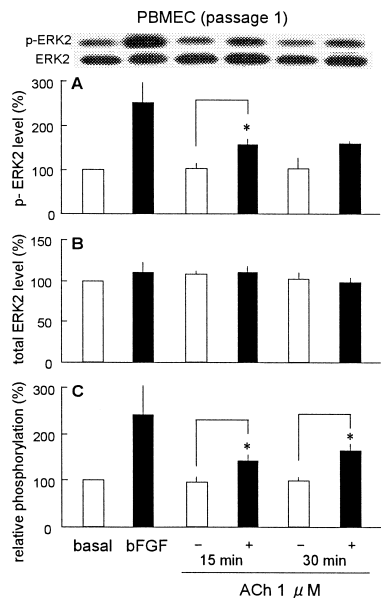


Fig. 6. Nicotinic Stimulation-Induced Phosphorylation of ERK2 in PBMEC

Serum-starved PBMEC (passage 1) were treated with atropine (1 μM) for 20 min. Subsequently, the cells were treated with either bFGF (30 ng/ml) for 10 min or acetylcholine (1 μM) for the indicated times, and then lysed for Western blotting analysis. Blots shown are representative of three independent Western blotting analysis demonstrating the effect of acetylcholine (ACh, 1 μM) on the phosphorylated ERK2 (p-ERK2) level and the total ERK2 expression level. Graphs shown are p-ERK2 level (A), total ERK2 level (B), and relative phosphorylation level of ERK2 in PBMEC (C). The relative phosphorylation level was calculated as the ratio of the density of the p-ERK2 band to that of the total ERK2 band in each sample. Data were expressed as percentages of the basal level (basal) obtained before stimulating by bFGF or acetylcholine in each experiment. Then, the results were averaged and presented as means ± S.E. ( $n=3$ ). \* $p<0.05$  compared with the levels in the non-stimulated cells at each time-point.

min, the phosphorylation levels were 1.5-fold and 1.7-fold increased, respectively, by acetylcholine (1 μM), compared with the phosphorylation levels in the non-stimulated cells at each time-point. The total ERK2 levels were not changed by these treatments (Fig. 6B). Similar results were obtained by treatment with nicotine (10, 1000 nM) for 30 min in the serum-starved PBMEC (data not shown).

To investigate whether the ERK/MAP kinase mediates the nicotinic enhancement of endothelial cell proliferation, the effect of PD98059, a MEK inhibitor, was examined in the PBMEC at passage 1. The cell numbers at day 8 were not altered by PD98059 (2 μM) alone (Fig. 7), although the basal cell proliferation under 10% serum-containing medium was significantly inhibited by PD98059 at a higher concentration (10 μM) (data not shown). The application of acetylcholine (1 μM) with atropine (1 μM) caused the 1.4-fold increase in the cell numbers in the absence of PD98059, but this enhancing effect of acetylcholine disappeared in the presence of PD98059 (2 μM).

## DISCUSSION

The present study provides the first evidence that nicotinic stimulation promoted the proliferation of cerebral microvascular endothelial cells *via* nicotinic receptor activation. The nicotine concentrations required to stimulate the BBMEC proliferation (0.1–1000 nM) correspond to those to stimulate the proliferation of peripheral endothelial cells derived from bovine pulmonary artery, human umbilical vein and human

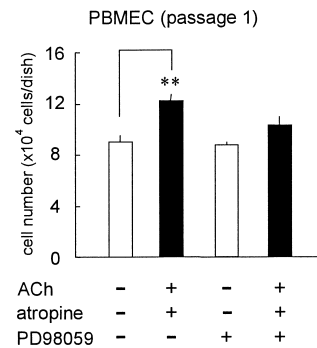


Fig. 7. Involvement of MAP Kinase in Nicotinic Receptor-Mediated Enhancement of PBMEC Proliferation

Effects of PD98059 (2 μM), a MAP kinase kinase inhibitor, on the proliferation of PBMEC (passage 1) in the presence (black column) and absence (open column) of acetylcholine (ACh, 1 μM) associated with the muscarinic antagonist atropine (1 μM) were examined at day 8 in culture. The number of cells was counted to address the activity of cell proliferation. All values are mean ± S.E.,  $n=8$  per group. \*\* $p<0.01$  compared with control response without drugs.

coronary artery.<sup>8,9</sup> Since the nicotine concentrations in the plasma of habitual smokers increase to these levels (<500 nM),<sup>21</sup> smoking appears to deliver a significant amount of nicotine to the brain with regard to cerebral angiogenesis. Although the stimulative effect of nicotine was relatively weaker comparing with that of an angiogenic growth factor FGF in the present conditions, repetitive and/or chronic application of nicotine might have some impact on the cerebral angiogenic activities *in vivo*.

The RT-PCR experiment was carried out using primers with which the transcripts of α3, α5, α7 and β4 nicotinic receptor subunits have previously been detected in bovine adrenomedullary chromaffin cells.<sup>15,16</sup> The present study demonstrated that BBMEC expressed the α3 transcript, whereas the α5, α7 and β4 transcripts were not detected. A recent immunoblot study using nicotinic receptor subunit-specific antibodies shows that α3, α5, α7, β2 and β3 subunits of nicotinic receptors are expressed in primary cultured BBMEC, whereas α4 and β4 subunits are undetectable.<sup>7</sup> Amounts of the α5 and α7 transcripts in BBMEC may not reach the detectable level in the present condition. On the other hand, slightly different expression patterns have been reported in rat coronary microvascular endothelial cells (α2, α3, α4, α5, α7, β2 and β4 subunits)<sup>22</sup> and in human aortic endothelial cells (α3, α5, α7, β2 and β4 subunits).<sup>5,6</sup> In contrast to the cerebral endothelial cells, these peripheral endothelial cells seem to express the β4-containing nicotinic receptor which is less sensitive to desensitization than the β2-containing and the α7-containing nicotinic receptors.<sup>23,24</sup> Thus, different pharmacological functions are predicted between cerebral and peripheral endothelial cells in chronic nicotinic stimulation.

It is noteworthy that BBMEC were highly sensitive to nicotine at early (less than four times) passages. Since the expression level of α3 transcript was remarkably decreased in BBMEC at passage 4, we speculate that nicotine fails to stimulate the proliferation via the α3-containing nicotinic receptor at this passage. A rescue treatment by overexpression of α3 nicotinic receptor would be useful to elucidate whether its decreased expression is responsible for a loss of nicotinic effect.

There were optimal nicotine concentrations (1–10 nM) to

stimulate the BBMEC proliferation, since less potent effect was produced by a higher nicotine concentration (1  $\mu$ M). These pharmacological profiles were the same as those in the peripheral endothelial cells.<sup>8,9)</sup> The weakened effect of nicotine at higher concentrations might be attributed to action of an inflammatory cytokine, tumor necrosis factor (TNF)- $\alpha$ , because nicotine induces the endothelial secretion of TNF- $\alpha$  which can mediate growth retardation in an autocrine manner in HUVEC.<sup>25)</sup>

The activation of the ERK/MAP kinase pathway promotes gene expression, cell proliferation and cell survival.<sup>26)</sup> We found that the phosphorylation of ERK2 was induced by nicotine in BBMEC, and was similarly induced by either nicotine or combination of acetylcholine+atropine in PBMEC. These results suggest that nicotinic receptors are equally activated by nicotine in both BBMEC and PBMEC. The nicotinic enhancement of PBMEC proliferation was blocked by a MEK inhibitor PD98059 at relatively low concentration that had no effect on the basal proliferation activity. The MEK inhibitor has been reported to block nicotine-induced capillary network formation by HUVEC.<sup>10)</sup> Thus, the ERK cascade seems to mediate the nicotinic modulation of both cerebral and peripheral angiogenic processes. Although nicotine induced the ERK2 phosphorylation in both BBMEC and PBMEC, the growth assays reveal the different susceptibility to nicotine between BBMEC and PBMEC. These raise a possibility that nicotine also affects downstream signaling molecules of ERK or other signaling pathways with different efficacy in BBMEC and PBMEC; as a result, nicotine may differently affect the proliferative activities of these endothelial cells. Indeed, in PC12 neuronal cells, nicotine induces the activation of both phosphatidylinositol 3-kinase/Akt and ERK pathways *via* common pathways including non- $\alpha$ 7 nicotinic receptors, L-type voltage-operated Ca<sup>2+</sup> channels and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II but Src family tyrosine kinases only participate in the nicotinic modulation of Akt.<sup>27)</sup> Further studies are required to elucidate the mechanism of functional coupling between nicotinic receptors and the ERK or other signaling pathways in the endothelial cells including cells derived from human brain microvascular endothelium.

The cerebral microvessels have a typical ultrastructure crucial to execute blood-brain-barrier function. To focal ischemia, the cerebral microvasculature rapidly displays multiple responses, such as breakdown of the blood-brain-barrier and the appearance of receptors associated with angiogenesis.<sup>28)</sup> Administration of nicotine is shown to reduce the barrier function, and decrease the expression of a tight-junctional protein, zonula occludens-1, in endothelial cells.<sup>7,29)</sup> Taken together with our findings, nicotinic input onto the microvessels seems to be one of the trigger signals in the cerebral angiogenesis, which could enhance the actions of angiogenic factors such as bFGF and vascular endothelial growth factors. Thus, nicotinic receptors may contribute to the formation of new capillary blood vessels that aid neurons with protection against brain ischemia.

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