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Gene Expression of Neurotrophins and Their Receptors in Cultured Rat Vascular Smooth Muscle Cells

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Most previous researches on neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) have focused on the nervous system, because their receptors are widely distributed in neuronal tissues. Recently, however, the participation of neurotrophins in inflammation and atherosclerosis has been proposed. Therefore, the gene expression of neurotrophins is now an urgent issue is to be investigated in nonneuronal tissues. Here, we evaluated the gene expression of neurotrophins and their receptors in rat cultured vascular smooth muscle cells (VSMCs) by the reverse transcriptase-polymerase chain reaction method. The transcripts of NGF, NT-3, and TrkC (high-affinity receptor for NT-3), and two BDNF alternative spliced transcript variants with exons 3 and 4 were clearly detected in VSMCs cultured under conventional culture conditions. The upregulation of mRNA levels for NGF, two BDNF variants with exons 1 and 2, low-affinity neurotrophin receptor, and high-affinity receptors, TrkA (for NGF) and TrkB (for BDNF), was observed in response to the treatment with serum and phorbolester following the serum-starvation. In contrast, the expression of NT-3 and TrkC genes was downregulated under these conditions. Co-expression of these factors and their receptors and the characteristic regulation of their gene transcriptions suggest that these factors play crucial roles in the function of VSMCs through an autocrine mechanism.

Neurotrophins include nerve growth factor (NGF) (1), brain-derived neurotrophic factor (BDNF) (2), neurotrophin-3 (NT-3) (3) and NT-4/5 (4). These neurotrophins function by binding to two types of cell-surface receptors, Trk tyrosine kinase receptors (5) and low-affinity neurotrophin receptor (LANR) belonging to the tumor necrosis factor receptor family (6). The Trk family is composed of three related members, TrkA which binds to NGF, TrkB which binds to BDNF and NT-4/5, and TrkC which binds to NT-3. These receptors are abundantly expressed in widespread neuronal populations. Therefore, most previous investigations of neurotrophins have mainly focused on their functions in the central and peripheral nervous systems. In fact, these studies have provided evidence that neurotrophins play crucial roles in the regulation of neuronal differentiation, maturation, and survival.

Cordon-Cardo *et al.* have reported that enforced expression of TrkA in fibroblast cells allows NGF to act as a potent mitogen on the fibroblast cells (7), suggesting for the first time that neurotrophins have the ability to exert a trophic function on nonneuronal cells which endogenously express Trk receptors. Recently, progress has been made in understanding the function of neurotrophins outside the nervous system. For example, NGF stimulates the proliferation of keratinocytes through binding to TrkA and LANR expressed in the cells (8). The co-expression of NGF, TrkA, and LANR is observed in B and T lymphocytes, suggesting that NGF is an autocrine survival factor for these cells (9, 10). BDNF may also be a survival factor for immature thymocytes as indicated by the expression of TrkB during T cell development (11). It has been reported that NT-3 is an essential factor in cardiac development (12, 13), which is supported by severe cardiac defects in TrkC-mutant mice (14). Additionally, cellular localization of the Trk family in nonneuronal tissues has been demonstrated (15, 16).

NGF released from vascular smooth muscle cells (VSMCs) has hitherto been considered to be a retrograde-transported trophic factor for sympathetic neurons innervated to blood vessels (17-19). Recently, Donovan *et al.* have observed the induction of not only NGF but also BDNF and Trk members in VSMCs that have

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migrated into the tunica intima in response to balloon-injury of the rat artery and the ability of NGF to stimulate migration of VSMCs *in vitro* (20). These findings for the first time raised the possibility that neurotrophins play crucial roles in regulating the response of VSMCs to injury through an autocrine/paracrine mechanism just like basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) (21, 22). Therefore, a detailed evaluation of gene expression for neurotrophins and their receptors in VSMCs is needed to test this hypothesis.

In the present study, we assessed the gene expression of neurotrophins (NGF, BDNF, and NT-3) and their receptors (LANR and Trk family) in rat cultured VSMCs treated with serum or phorbol-ester, TPA (12-*O*-tetradecanoyl phorbol-13-acetate), both of which promote the proliferation of these cells. The results demonstrate that the expression patterns of all genes respond to both treatments in a characteristic manner, supporting the hypothesis that neurotrophins exert some important functions in VSMCs through the autocrine and/or paracrine mechanisms. At the same time, the cultured VSMCs were illustrated to be a useful *in vitro* system for investigating the expression mechanisms of neurotrophins and their receptors outside of the nervous system.

MATERIALS AND METHODS

Preparation of VSMCs and treatment with serum or TPA. VSMCs were isolated by enzymatic digestion from the thoracic aortic media of 14-week-old Wistar-Kyoto (WKY/Izm) rats (Disease Model Cooperative Research Association) using a mixture of collagenase (Wako Pure Chemicals Industries Ltd, Tokyo, Japan), elastase (Sigma), and trypsin inhibitor (Sigma) as described previously (19). Cells were normally cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO_2. For estimating the effects of serum and TPA on gene expression, cells were grown in DMEM supplemented with 10% FCS, until nearly confluent. The cells were then serum-starved by maintaining them in DMEM containing 0.5% FCS. Two days later, the cells were treated either with FCS to a final concentration of 20% or with TPA to a final concentration of 100 ng/ml.

RT-PCR. Total RNAs were prepared by ISOGEN (NipponGene, Japan) from cells at appropriate time points after serum- or TPA-treatment. The gene expression levels of neurotrophins, their receptors, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (as an internal control) genes in cells with varying treatments were determined by RT-PCR. A 4 μg portion of total RNA was converted into cDNA using polyd$(N)_6$ primer (Pharmacia Biotech) and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) in a total reaction volume of 20 μl (RT-reaction mixture). PCR was performed using one twenty-fifth volume of the RT-reaction mixture, 0.5 μM of each primer (forward and reverse primers), and AmpliTaq DNA polymerase (PERKIN ELMER) in a total reaction volume of 25 μl. The primers used for PCR were located at the positions shown in Fig. 1. To detect four BDNF alternatively spliced transcripts, four primer sets were used; exon 1 specific: 5′-ACTCAAAGGGAAACGTGTCTCT-3′ (forward), exon 2 specific: 5′-CGGTGTAGGCTGGAATAGACT-3′ (forward), exon 3 specific: 5′-CTCCGCCATGCAATTTCCACT-3′ (forward), exon 4 specific: 5′-GTGACAACAATGTGACTCCACT-3′ (forward), and exon 5 specific: 5′-TCGATCACGTGCTCAAAAGTGT-3′ (reverse). To detect two NT-3 alternatively spliced transcripts, two sets of the following primers were used; NT3E1A; 5′-CAAACCTCCAAAGTGCTGTGT-3′ (forward), NT3E1B; 5′-CCCTGGAAATAGTCATACGGAT-3′ (forward) and NT3R; 5′-GGGGTGAATTGTAGCGTCTCT-3′ (reverse). As primers for rat NGF, LANR, Trk A, TrkB, TrkC, c-*fos*, c-*jun*, and GAPDH transcripts we used 5′-TGGACCCAAGCTCACCTCA-3′ (forward) and 5′-GTGGATGAGCGCGCTTGCTCCT-3′ (reverse), 5′-CCTCTGGAGGTGCCAAGGAG-3′ (forward) and 5′-TTGGATCCTGCTGGGCGCTGTGCTGTC-3′ (reverse), 5′-TGGCTGCCTTCGCCTCAACCAG-3′ (forward) and 5′-ATGGTGGACACAGGTATCACTG-3′ (reverse), 5′-ATTGACCCAGAGAACATCAC-3′ (forward) and 5′-CAGGAAATGGTCACAGACTT-3′ (reverse), 5′-CCCTACACCTCCTATCACTG-3′ (forward) and 5′-CTGGAAATCCTTCCTGGCAG-3′ (reverse), 5′-CACGACCATGATGTTCTCGG-3′ (forward) and 5′-AGTAGATTGGCAATCTCGGT-3′ (reverse), 5′-TGAAGCAGAGCATGACCTTG-3′ (forward) and 5′-GACACTGGGCAGCGTATTCT-3′ (reverse), and 5′-TTCAACGGCACAGTCAAGG-3′ (forward) and 5′-CATGGACTGTGGTCATGAG-3′ (reverse), respectively. The amplification protocol consisted of 25 to 40 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and extension for 2 min at 72°C. The PCR products were visualized by ethidium bromide staining under UV light following electrophoresis on a 2% agarose gel.

RESULTS AND DISCUSSION

We assessed the gene expression patterns of neurotrophins and their receptors in VSMCs prepared from rat thoracic aortic media by the RT-PCR method. Alternative splicing of 5′ exons has been observed in all neurotrophin genes (23-25) (Fig. 1A). It has been reported that each transcript variant for BDNF and NT-3 genes is regulated by a specific promoter. We therefore used the primer sets for PCR to distinguish each transcript variant. In the NGF transcripts, a primer set was used to detect all transcript variants. In members of the Trk family, insertion of polypeptides or truncation in the carboxy-termini have been observed (5) (Fig. 1B). In the present study, we used the primer sets to detect all types except the truncated Trk members.

The expression levels of these genes in VSMCs cultured under conventional culture conditions (in 10% FCS) are shown in Fig. 2. The transcripts for NGF, NT-3, LANR, and TrkC genes were detectable at 35 cycles of PCR, while the transcripts for TrkA but not TrkB genes was detected at 40 cycles (Fig. 2A). As for the BDNF gene, transcripts with exons 3 and 4 were strongly detected at 30 to 35 cycles, while transcripts with exons 1 and 2 could be detected at 40 cycles.

We estimated the expression of these genes in response to serum- or TPA-treatments (Fig. 3). NGF gene expression was induced by each treatment and peaked at 2-4 hr. This pattern was identical to typical patterns as previously demonstrated in various cultured cell lines treated with cytokines, growth factors, and second messenger activators (26-29). The BDNF transcript with exon 1 also became detectable from 2 hr after the onset of each treatment, peaked at 4 hr, and then decreased. This level of response was higher with TPA-treatment than the serum-treatment. Expression of the BDNF transcript with exon 2 appeared to be induced at

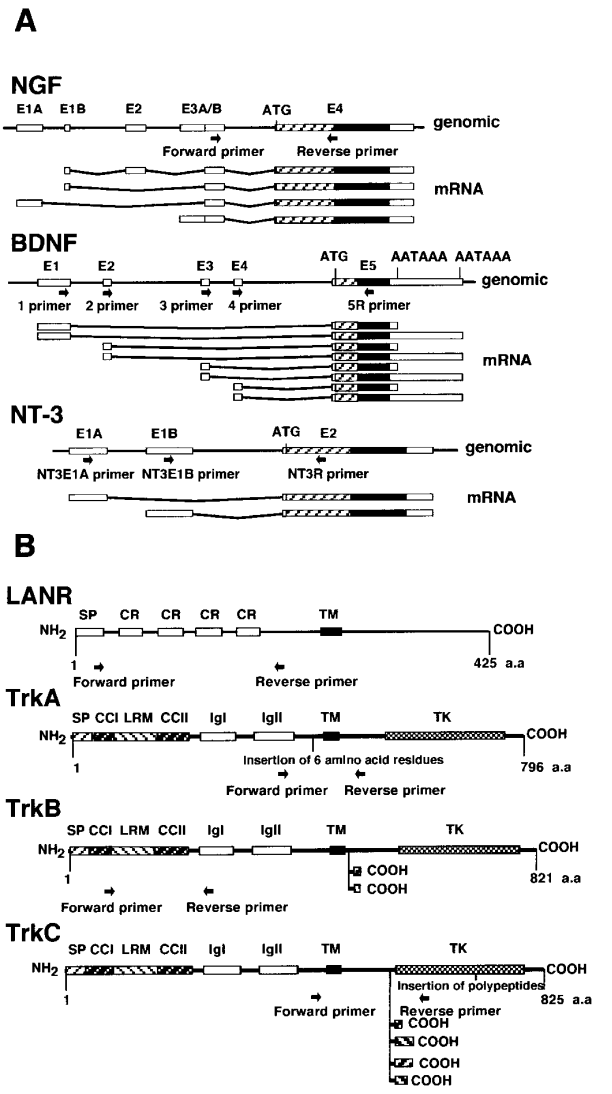

FIG. 1. Schematic representation of neurotrophin genes (A) and neurotrophin receptors (B). In panel A, the exon (E) regions are indicated by boxes; especially, prepro and mature protein-coding regions are indicated by hatched and filled boxes, respectively. In panel B, SP, CR, CC (I and II), LRM, Ig (I and II), TM, and TK represent the structural domains of the signal peptide, cysteine-rich region, cysteine clusters, leucine-rich motif, immunoglobulin-like C2-type motifs, the transmembrane region, and the tyrosine kinase catalytic domain, respectively. The positions of primers for RT-PCR used in our studies are indicated by arrows. These figures were adapted from reports by Selby *et al.* (25), Timmusk *et al.* (23), Leingartner and Lindholm (24), Radeke *et al.* (52), and Barbacid (5).

2-4 hr after each treatment. As for the BDNF transcript with exons 3 and 4, weak induction could be observed at 1 hr at lower cycles (30 and 25 cycles, respectively) of PCR because of their predominant expression even under the conventional culture condition (Fig. 2). It has been demonstrated that alternatively splicing transcriptions of the BDNF gene are regulated in a tissue- and stimulation-specific manner (23, 30). In particular, expression of the transcripts with exons 1 and 2 has been observed only in the brain. Induced BDNF expression in response to serum and TPA has already been reported in various nonneuronal cultured cells (31-33). However, these approaches were performed without distinction of the four transcript variants. Therefore, our data has demonstrated for the first time the possibility of their different expression in nonneuronal cells. Recently, in hippocampal neurons, it has been reported that transcripts with exons 3 and 4 are induced with the characteristics of immediate early genes (IEGs) following seizures, while increased expression of transcripts with exon 1 and 2 are dependent on a prior step involving protein synthesis (34). Judging from the temporal expression patterns, expression of the BDNF transcripts in response to serum and TPA in VSMCs seems to also be regulated in such a characteristic manner. The expression patterns of NT-3 and receptors in response to serum and TPA have not yet been reported. The levels of two NT-3 transcripts were markedly reduced at 2 hr. The level for the LANR transcript markedly increased from 2 hr after each treatment, peaked at 4 hr, and then decreased. Its induced level in response to serum-treatment is higher than in response to TPA-treatment. TrkA gene expression was induced 8 hr after each treatment, although the effect of the serum-treatment appeared to be stronger than that of TPA-treatment. TrkB gene expression was induced at 1 hr by serum-treatment, while higher induction by TPA-treatment peaked at 2 hr. The level for TrkC transcript in response to serum-treatment was reduced after 4 hr, while its expression in response to TPA-treatment was reduced after its induction at 1 hr. We also evaluated the expression of c-*fos* and c-*jun* genes, both of which encode the component of AP-1 transcription factor (35). Expression of the c-*fos* and c-*jun* genes in VSMCs was super-induced from 0.5 to 1 hr after serum- and TPA-treatment as previously reported in many cultured cell types (36).

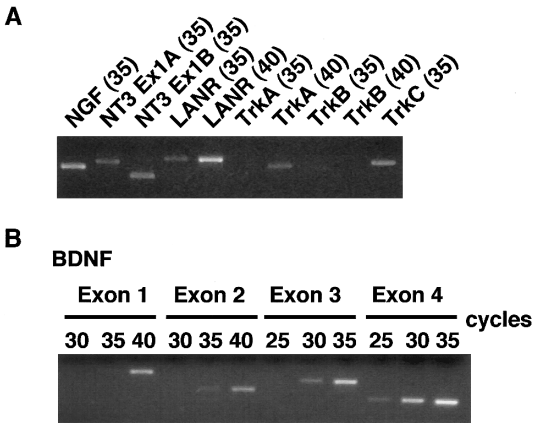

FIG. 2. Gene expression of neurotrophins and their receptors in VSMCs cultured under conventional culture conditions. In panel A, numbers in the brackets indicate the number of cycles set in PCR.

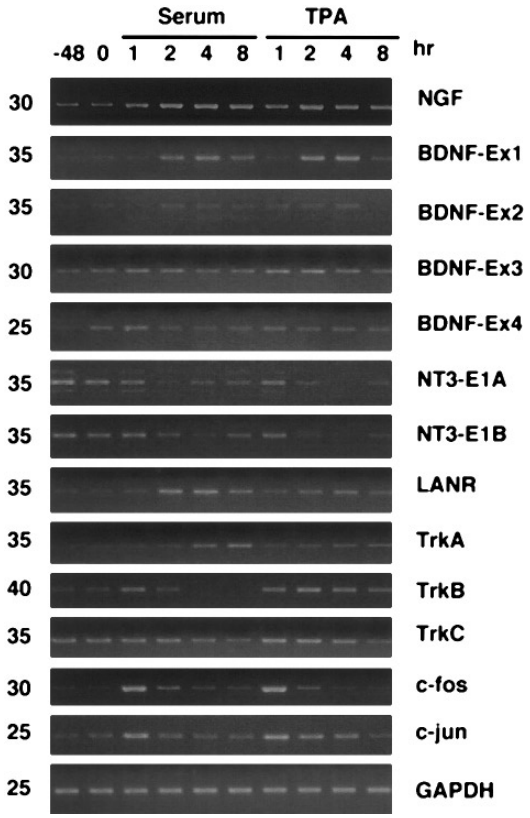

FIG. 3. Gene expression of neurotrophins, their receptors, c-*fos* and c-*jun* in VSMC treated with serum or TPA. Lane -48 (hr) indicates the expression of transcripts in VSMC at the onset of serum-starvation. Numbers on the left side of the photograph indicate the number of cycles set for PCR.

We have reported here the expression patterns of neurotrophins and their receptors in cultured VSMCs under conventional culture conditions and when treated with serum or TPA by estimating the levels of their transcripts. Smooth muscle cells generally have two typical phenotypes *in vivo; i.e.* differentiated phenotype with contractility, and dedifferentiated phenotype with proliferation and migration abilities. A converse transition of VSMCs from a differentiated to dedifferentiated phenotype occurs during the formation of vascular lesion including atherosclerosis (37, 38) and the early *in vitro* culturing process (39, 40). The VSMCs used in our culturing system and that migrate into the tunica intima in response to balloon-injury of rat artery are considered to be dedifferentiated. Therefore, the expression patterns indicated in our system are expected to reflect those during the proliferation and migration process of VSMCs in the vascular lesion area as previously reported by Donovan *et al.* (20). In the present study, we first demonstrated that VSMCs express NGF, BDNF, NT-3, TrkA (receptor for NGF), TrkC (receptor for NT-3), and LANR genes under conventional culture conditions. Secondly, we observed that the levels of transcripts for NGF, BDNF (especially with exons 1 and 2), LANR, TrkA, TrkB (receptor for BDNF), and LANR are induced by the treatment of VSMCs with serum or TPA following serum-starvation. Considering the expression patterns of the receptors, it is speculated that NGF and NT-3 maintain the normal growth of cultured VSMCs through an autocrine/paracrine mechanism and that NGF and BDNF further promote the proliferation initiated by either agent through an autocrine/paracrine mechanism. Their temporal variation is also likely to be important in the proliferation and migration of VSMCs following vascular injury as well as in the proliferation of cultured VSMCs. However, both treatments attenuated the expression of NT-3 and TrkC. This may suggest that NT-3/TrkC have roles that are different from those of NGF and BDNF in the proliferation of VSMCs. Dedifferentiation of VSMC is a fundamental response to vascular injury and is important in the pathogenesis of hypertensive vascular disease, atherosclerosis, and restenosis after balloon angioplasty (41, 42). We therefore propose that the relationship between neurotrophins and the expression of VSMC phenotypes be further investigated in order to understand this kind of pathogenesis.

Of the genes assessed here, the nucleotide sequences of the proximal regions of the NGF (43), BDNF (23, 44), NT-3 (24, 45), LANR (46), and TrkA (47) genes have been determined. The expression of the NGF gene has already been proven to be upregulated by the binding of AP-1 to TRE (TPA response element) in the first intron of its gene (43). The LANR promoter has a cell type-specific negative regulatory element containing E-box sequences (48). Recently, SP1 and Wilms' tumor suppressor WT1 proteins have been demonstrated to be positive and negative factors, respectively, for LANR gene expression (49). As for the other three genes, however, though there appear to be several consensus sequences, including TRE, for transcriptional factors in their promoter regions, the actual function of these sequences remains to be clarified. We have demonstrated here that each gene is expressed with a characteristic temporal expression pattern in response to serum- and TPA-treatments. Additionally, as for some of the evaluated transcripts, the responding levels differ according to the treatment; *i.e.*, the expression levels of BDNF with exon 1 and TrkB transcripts are higher in response to TPA-treatment than to serum-treatment, while those of LANR and TrkA transcripts respond in an opposite manner. Serum-treatment leads to a gradual reduction of TrkC transcript levels, while the levels are reduced at 1 hr after TPA-treatment. Treatments of cultured cells with serum and TPA give rise to the qualitative/quantitative activation of several transcriptional factors including serum response factor (SRF) (50) and AP-1 (51), which recognize the consensus sequences of SRE (serum response element) and TRE, respectively. These characteristic expression patterns may provide information that may elucidate the tran-

scriptional mechanisms not only in VSMCs but also in other nonneuronal and neuronal cells.

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