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Neurotransmitter noradrenaline downregulate cytoskeletal protein expression of VSMCs

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ABSTRACT

Objective: This study investigates the effects of noradrenaline (NA) on cytoskeletal protein expression of vascular smooth muscle cells (VSMCs).
Methods: VSMCs were isolated from rat aortic tissue and cultured. The cultured VSMCs were divided into 4 experimental groups: (1) control group, (2) NA treatment group, (3) starvation group, and (4) NA treatment + starvation group. The expression of cytoskeletal protein (smooth muscle α-actin, β-tubulin and desmin) was evaluated by (i) Coomassie blue staining, (ii) immunofluorescent staining, and (iii) RT-PCR and Western Blot.
Results: NA treatment significantly downregulated the expression of SM α-actin, β-tubulin and desmin ($P<0.05$). The serum starvation did not affect the expression of cytoskeletal protein (SM α-actin, β-tubulin and desmin), but when the cells were treated with the combination of NA and serum starvation, the expression of SM α-actin, β-tubulin and desmin were down-regulated than those of the serum starvation group ($P<0.05$).
Conclusion: These results suggested that NA might play a key role in regulating the cytoskeletal protein expression of VSMCs.

Introduction

The proliferation of vascular smooth muscle cells (VSMCs) and their migration from media to intima play important roles in the pathogenesis of vascular diseases such as atherosclerosis and restenosis after angioplasty (Abedi and Zachary, 1995). Previous studies have shown that cell migration is regulated by the cytoskeleton proteins such as SM α-actin, β-tubulin and desmin (Gerthoffer and Gunst, 2001). Cytoskeleton is a complex network of filaments and tubules that transmits mechanical and chemical stimuli within and between cells. It contributes substantially to cell stability by anchoring subcellular structures such as mitochondria, Golgi apparatus, nuclei, as well as myofibrils. Cytoskeleton acts as a stabilizing force and mechanotransductor that are supported by membrane-associated proteins, especially dystrophin that binds to both intracellular actin and extracellular laminin (Dietzmann et al., 2003; Stefan et al., 2000; Wang et al., 2007). Thus, the cytoskeleton organization of VSMCs plays a key role in vascular remodeling-associated diseases such as atherosclerosis and hypertension.

It is known that vasoconstriction can be regulated by the sympathetic nervous system where noradrenaline (NA) is one of the major neurotransmitters. NA binds to the membrane receptors of VSMCs leading to vasoconstriction (Kacem and Sercombe, 2006). It remains to be determined whether the sympathetic nervous neurotransmitter NA can regulate the cytoskeletal protein expression of VSMCs. In our previous study, we demonstrated that NA could promote proliferation and phenotypic transformation of VSMCs (Jiao et al., 2008). To further understand the underlying mechanism between cytoskeleton and VSMCs proliferation, current study investigated the effects of sympathetic neurotransmitter NA on the expression of cytoskeleton proteins of the VSMCs. We showed, for the first time, that NA induced reorganization of the actin cytoskeleton and downregulated the expression of SM α-actin, β-tubulin and desmin. Our results suggested a critical role for sympathetic neurotransmitter NA in cytoskeleton organization of VSMCs, which may provide a potential novel targeting strategy for inhibiting VSMC proliferation via modulating nervous function.

Materials and methods

Animals and reagents

Sprague–Dawley (SD) rats were purchased from the Experimental Animal Center of Suzhou University (Suzhou, China), weighing 150–200 g. The experimental protocol was approved by the Ethics Committee of Medical College, Suzhou University. The reagents used include: newborn bovine serum (NBS) from Every Green Co. (Hangzhou, China); Dulbecco's modified Eagle's medium (DMEM) and trypsin from Gibco

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Inc. (Carlsbad, CA, USA); anti-desmin antibody, and anti-alpha-actin antibody from Boster Biotech. (Wuhan, China); noradrenaline, phalloidin-FITC and anti-tubulin antibody from Biosynthesis Biotechnology Co. (Beijing, China); polyvinylidene fluoride (PVDF) membrane from BIO-RAD Laboratories Ltd. (Beijing, China), Immuno-Star Kit from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Chemiluminescent Detection Kit from Amersham (Piscataway, USA).

VSMC culture and treatment

The abdominal aortas were isolated from the SD rats under aseptic conditions. After the aortic intima was peeled off, the media was then carefully stripped away from the adventitia and placed in a petri-dish containing warm *N*-hydroxyethylpiperazine-*N*-ethanesulfonate (HEPES)-buffered DMEM (37 °C). The media was cut into pieces of approximately 1 mm^3, which were then transferred to a 25-cm^2 tissue culture disk and incubated with DMEM supplemented with 20% NBS. After 2 weeks, the cells were trypsinized and subcultured successively 3 times. The VSMC nature of the prepared cells was validated by immunocytochemistry staining with anti-SM α-actin antibody (Jiao et al., 2008). Cells were used for the experiments after three passages, and were cultured in the growth medium containing 0.5% NBS for 24 h in order to synchronize the cell cycles before treatment. The experimental groups included that treated with 10% NBS media (control group), 10% NBS media containing 50 μM of NE (NE treatment group), serum-free media (starvation group), or serum-free media containing 50 μM of NA (NA + starvation group), and the cells were treated for another 48 h.

Immunofluorescent staining

Immunofluorescent staining for cytoskeleton proteins was performed as described below: 1) The coverslips were incubated in methanol containing 0.4% H_2O_2 at room temperature for 30 min to block endogenous peroxidase activity; 2) treated with 0.3% Triton X-100 in PBS for 30 min; 3) incubated with blocking medium (10% normal goat serum) for 30 min at room temperature; 4) incubated with primary antibody against SM α-actin, desmin, tubulin and PCNA diluted in PBS (1:500) overnight at 4 °C; 5) incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG 568 (1:500) for 30 min at room temperature; 6) rinsed in 0.01 M PBS three times between steps; and 7) mounted with liquid paraffin and observed under the fluorescence microscope. The immunofluorescent experiment was repeated for 3 times.

All of the data were analyzed using a computation imaging analysis system (CMIAS). In each group, the integrated optical density (IOD) of five coverslips was analyzed using SigmaScan 5.0 software. Statistical analysis was performed using the *t*-test. Differences were considered statistically significant when $P<0.05$.

RT-PCR

Total RNA was isolated from the VSMCs with Trizol reagent. RNA purity was assessed by spectrophotometry (A260/A280 > 1.8). cDNA was synthesized from 1 μg of total RNA in a 20 μl reverse transcription (RT) system followed by PCR amplification in a 50-μl PCR system performed using an RT-PCR kit according to the manufacturer's instructions. The following primers were used: SM α-actin (265 bp): 5′-ACTGGGACGACATGGAAAAG-3′ (sense) and 5′-TAGATGGGGACA TTGTGGGT-3′ (antisense); β-tubulin (647 bp): 5′-ATCTGCTTCCGCA CCCTC-3′ (sense) and 5′-CTCGTCGTCATCTTCATACATC-3′ (antisense); desmin (330 bp): 5′-GAAGTAACAAGCCTGTCTTG-3′ (sense) and 5′-GATCTCTCTTGCCCACTAGC-3′ (antisense); and GAPDH (309 bp): 5′-TCCCTCAAGATTGTCAGCAA-3′ (sense) and 5′-AGATCCACAACGGA TACATT-3′ (antisense). RT-PCR was performed using a kit from Promega (Madison, WI, USA) and GAPDH was used as an internal standard. The PCR conditions were as follows: pre-denaturation at 95 °C for 5 min, followed by 32 cycles of amplification at 94 °C for 50 s, 56 °C for 50 s, 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 1.7% agarose gel followed by ethidium bromide staining. The target bands were analyzed densitometrically using a GIS 2010 image analyzer and the results were expressed as the ratio of the optical density (OD) value relative to that of GAPDH. All experiments were repeated for 3 times.

Western blotting analysis

VSMCs were lysed with a lysis buffer (50 mM Tris–HCl, 0.15 M NaCl, 0.15% Triton-X100, 1% SDS, 5 mM EDTA, 50 mM NaF, 3 μg/ml aprotinin, 2 mM PMSF, pH 7.2). After heat-denaturation with 2-mercaptoethanol, each sample (protein amount 15–20 μg) was subjected to 10% SDS-PAGE. The separated proteins in poly-acrylamide gels were electrophoretically transferred to a polyvinylidene fluoride (PVDF, BIO-RAD, USA), and immunostaining was performed using an Immuno-Star Kit (Wako Chemical, Japan). The membrane was incubated with 5% skimmed milk for 1 h at room temperature, and subsequently incubated overnight at 4 °C with α-actin, β-tubulin and desmin antibody, respectively. Immunodetection was carried out with a Chemiluminescent Kit (Amersham, USA), according to the manufacture's recommended protocol. The experiments were performed in triplicate and were repeated more than 3 times.

Statistical analysis

All values were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by unpaired Student's *t*-test with Fisher's protected least significant difference for multiple comparisons. $P<0.05$ was considered as statistically significant.

Results

VSMCs are known to express relatively high levels of cytoskeletal proteins such as SM α-actin, β-tubulin and desmin. To investigate whether noradrenaline affects the cytoskeleton of VSMC, cells were cultured in growth medium containing 0.5% NBS for 24 h and then stimulated with 50 μM of noradrenaline in the presence or absence of serum. Cells were fixed and then stained with Coomassie blue for visualizing the structure, or the antibodies for actin, desmin or tubulin to examine the expression of the cytoskeletal proteins. Coomassie blue staining showed that there were fewer stress fibers in cells treated with NA than those of cells treated with vehicle (Fig. 1A). Interestingly, the absence of NBS in the culture medium negated the effects of NA. Immunofluorescent staining further confirmed that there were decreased expressions of cytoskeleton proteins. As it was shown in Fig. 1B, there were significant less staining of SM α-actin, β-tubulin or desmin in VSMC treated with NA than that treated with vehicle. Likewise, absence of NBS attenuated the effects of NA (Fig. 1B and C).

Western blotting and RT-PCR were further performed to investigate whether NA affects the cytoskeleton proteins at the transcription or translation level. As it is shown in Fig. 2, noradrenaline alone downregulated the protein and mRNA expressions of cytoskeleton proteins SM α-actin, β-tubulin as well as desmin. The serum starvation alone did not affect the expression of all the cytoskeletal proteins examined, however, attenuated the NA-induced decrease in the expressions of cytoskeleton proteins (Fig. 2A and B).

PCNA is a non-histone nuclear protein commonly used as a 'proliferation marker' both in normal and disease states. Expression of PCNA is cell cycle-dependent and is required for progression of cells from G1-phase to S-phase. To investigate whether the changes of cytoskeleton protein expression was associated with changes in cell proliferation,