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Inhibitory effect of D_3 dopamine receptors on neuropeptide Y-induced migration in vascular smooth muscle cells

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Abstract. Abnormal migration of vascular smooth muscle cells (VSMCs) serves an important role in hypertension, atherosclerosis and restenosis following angioplasty, which is regulated numerous hormonal and humoral factors, including neuropeptide Y (NPY) and dopamine. Dopamine and NPY are both sympathetic neurotransmitters, and a previous study reported that NPY increased VSMC proliferation, while dopamine receptor inhibited it. Therefore, the authors wondered whether or not there is an inhibitory effect of dopamine receptor on NPY-mediated VSMC migration. The present study demonstrated that stimulation with NPY dose-dependence (10^{-10}-10^{-7}M, 24 h) increased VSMC migration, the stimulatory effect of NPY was via the Y_1 receptor. This is because, in the presence of the Y_1 receptor antagonist, BIBP3226 (10^{-7} M), the stimulatory effect of NPY on VSMC migration was blocked. Activation of the D_3 receptor by PD128907 dose-dependence (10^{-11}-10^{-8} M) reduced the stimulatory effect of NPY on VSMC migration. The effect of PD128907 was via the D_3 receptor, because the inhibitory effect of PD128907 on NPY-mediated migration was blocked by the D_3 receptor antagonist, U99194. The authors' further study suggested that the inhibitory effect of the D_3 receptor was via the PKA signaling pathway, in the presence of the PKA inhibitor, 14-22 (10^{-6} M), the inhibitory effect of PD128907 on VSMC migration was blocked. Moreover, the inhibitory effect of PD128907 was imitated by PKA activator, Sp-cAMP [S], in the presence of Sp-cAMP [S], the NPY-mediated stimulatory effect on VSMC migration was abolished. The present study indicated that activation of the D_3 receptor inhibits NPY Y_1-mediated migration on VSMCs, PKA is involved in the signaling pathway.

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Introduction

Abnormal migration of vascular smooth muscle cells (VSMCs) serves an important role in hypertension, atherosclerosis development and restenosis following angioplasty (1-3), which is regulated by numerous hormonal and humoral factors, including neuropeptide Y (NPY) and dopamine (4,5). NPY is a tyrosine-rich 36-amino acid peptide that was initially isolated from brain in the early 1980s (6). It is widely distributed in the central and peripheral nervous system (7). NPY has both potent central and peripheral biological effects, including cardiovascular actions, which participate in the development of cardiovascular diseases. There are also reports indicating that NPY receptor existed in VSMCs, and that stimulation of VSMCs with NPY increased VSMC proliferation and migration (8-10). In hypertension or atherosclerosis, NPY-mediated migration of VSMCs is augmented (11).

Dopamine is an endogenous catecholamine that regulates many cellular and physiological processes, including behavior, hormone synthesis and release, blood pressure, and transmembrane ion transport (12). Dopamine receptors have been divided into D_1- and D_2-like subtypes based on their interaction with the effector enzyme, adenylyl cyclase. D_1-like receptors that stimulate adenylyl cyclases, comprised of D_1 and D_5 receptors, whereas D_2-like receptors, comprised of D_2, D_3, and D_4 receptors, inhibit adenylyl cyclase activity and regulate/modulate the activity of several ion channels (13,14). Dopamine and NPY are both sympathetic neurotransmitters, but dopamine has a significant anti-hypertensive and anti-atherosclerotic effect (15,16). Previous studies of the authors reported that activation of dopamine receptor inhibits norepinephrine-mediated VSMC proliferation (10,17), a similar inhibitory effect of dopamine on NPY-mediated VSMC proliferation was also observed. The authors intended to understand whether or not there is an inhibitory effect of dopamine receptor on NPY-mediated VSMC migration. They demonstrated that, among all these dopamine receptor subtypes, the D_3 receptor inhibits NPY receptor mediated-VSMC migration; this inhibitory effect of D_3 receptor is via the PKA signaling pathway.

Materials and methods

Cell culture. VSMCs were isolated from the aortic strips of male Sprague-Dawley rats weighing 200-250 g with an

implant technique previously described (10). This experiment was approved by the Third Military Medical University Animal Use and Care Committee (Chongqing, China). Briefly, the arteries were dissected free of fat and excess adventitial tissue and then opened along its longitudinal axis, and the endothelial lining was removed by vigorously scraping of the lumen with a scalpel blade. Small fragments (~1 mm^3) were transferred to a flask and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% penicillin/streptomycin/bFGF/insulin/epidermal growth factor (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)] in a humidified atmosphere (95% air/5% CO_2) at 37˚C. The purity and identity of VSMCs were verified by their typical morphological pattern (multilayer sheets and 'hills and valleys') and by immunohistochemistry staining using specific mouse monoclonal antibody against smooth muscle cell α-actin (cat. no A5228; 1:100; Sigma-Aldrich; Merck KGaA) at 37˚C for 1 h. Cells from passages 3 to 5 were used for experiment. The cells were then placed in the medium without serum for 24 h to render them quiescent prior toexperiments (18,19).

Transwell migration assay. Cells were trypsinized and laid down on top of 8.0 μm nucleopore polycarbonate membrane Transwell inserts (6-well plates; EMD Millipore, Billerica, MA, USA) at a density of $3x10^5$ cells/well. The cells were then incubated for 24 h at 37˚C under 5% CO_2 and 95% air in a humidified incubator in limiting medium with or without NPY and supplemented with other reagents as indicated under 'Results'. Membranes were washed three times in PBS and the cells in the upper chamber were harvested using cotton swaps and thorough brushing. The migrated cells on the underside of the membrane were fixed in 4% paraformaldehyde at room temperature for 30 min and stained with 0.1% crystal violet at room temperature for 30 min and counted per three independent HPFs (magnification, x100) with light microscope (20,21).

Wound healing assay. VSMCs were plated at a density of $5x10^5$ /cm^2 in 6 cm-diameter culture dishes, and incubated at 37˚C in a CO_2 incubator for 12 h, allowing cells to completely adhere and spread on the dishes. The cells grew and were maintained in serum-free DMEM for >24 h to create a confluent monolayer. The confluent monolayer was scraped with a sterile 200 μl pipette tip and washed with PBS. Serum-free DMEM and indicated reagents were then added, and the distance between the two edges of the scraped area was measured under a light microscope (magnification, x10). Half of the difference between the two distances was regarded as the migratory distance of cells. Experiments were performed in triplicate (22,23).

Statistical analysis. The data are expressed as mean ± standard deviation. All data analysis was performed with SPSS statistical software (version, 13.0; SPSS Inc., Chicago, IL, USA). Differences between the two groups were compared using unpaired Student's t-tests. Multiple comparisons between the groups was performed using one-way analysis of variance, the post hoc analysis was performed by S-N-K method. P<0.05 was considered to indicate a statistically significant difference.

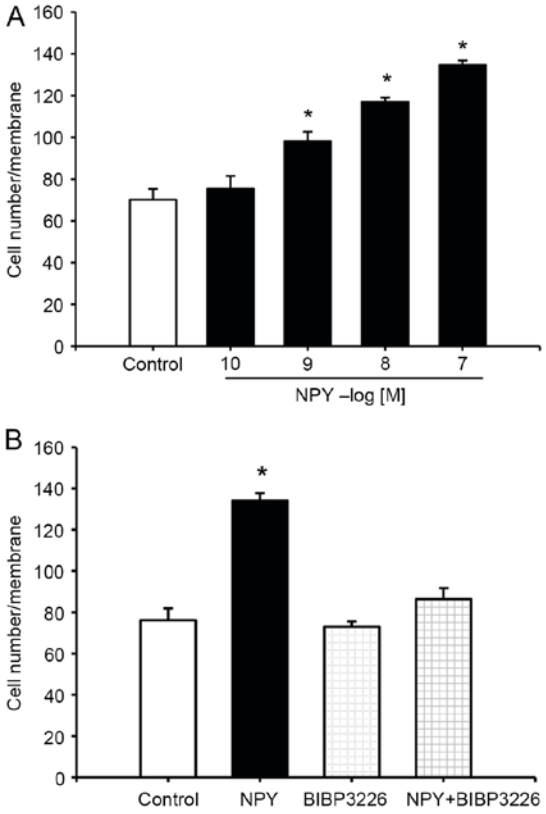

Figure 1. Effect of NPY on vascular smooth muscle cell migration. (A) Effect of varying concentrations of NPY (10^{-10}-10^{-7}M, 24 h) on vascular smooth muscle cell migration. *P<0.05 vs. control (n=6). (B) The NPY Y_1 receptor antagonist (BIBP3226, 10^{-7} M) significantly attenuates the increased migration induced by NPY (10^{-8} M). *P<0.05 vs. control (n=6). The data are expressed as mean ± standard deviation. NPY, neuropeptide Y.

Results

NPY, via Y_1 receptor, increases migration of VSMCs of aorta from SD rats. Treatment of VSMCs with varying concentrations of NPY (10^{-10}-10^{-7}M, 24 h) increased the migration of VSMCs from SD rats in a concentration-dependent manner (Fig. 1A). The increased migration, induced by NPY, was blocked by the NPY Y_1 receptor antagonist, BIBP3226, (10^{-7} M; Fig. 1B), as previous described (24), indicating that the NPY-mediated migration was via the NPY Y_1 receptor.

NPY-mediated VSMC migration is attenuated by D_3 receptor activation. To investigate whether there is an interaction between NPY Y_1 and D_3 receptors, VSMCs were incubated with NPY and the D_3 receptor agonist, PD128907 (10^{-11}-10^{-8}M, 24 h). PD128907 had no effect on migration in VSMCs (Fig. 2A), but dose-dependence (10^{-11}-10^{-8}M) reduced the stimulatory effect of NPY on VSMC migration (Fig. 2B). The effect of PD128907 was via the D_3 receptor, because the inhibitory effect of PD128907 on NPY-mediated migration was blocked by the D_3 receptor antagonist, U99194 (10^{-7}M; Fig. 2C). The scratch assay also indicated that the increased average migration distance induced by NPY, was also blocked by the D_3 receptor agonist, PD128907, while D_3 receptor antagonist U99194 (10^{-7}M) significantly blocked the inhibitory effect of PD128907 on NPY-mediated migration (Fig. 2D). Fig. 2E presents quantification of Fig. 2D.

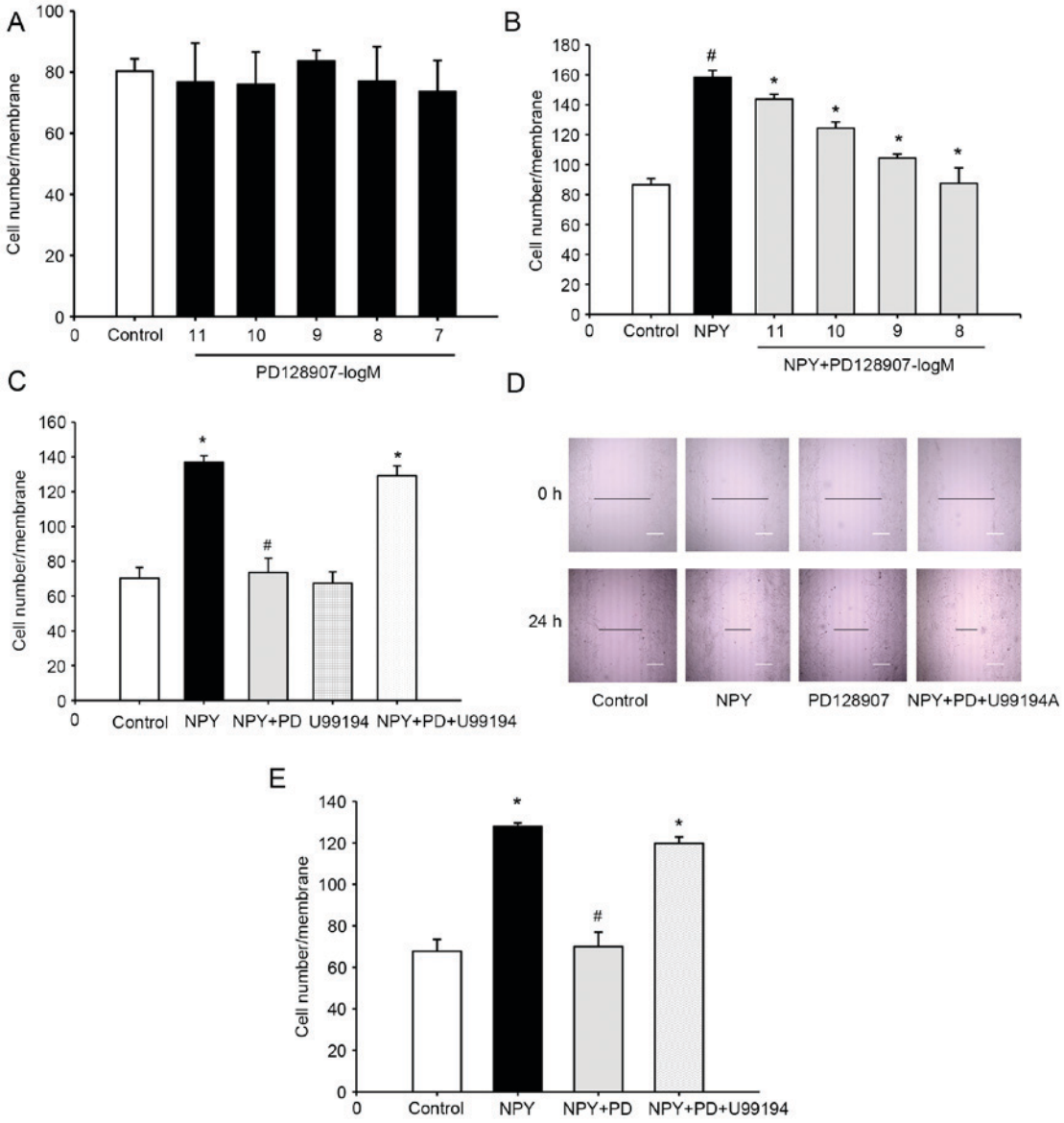

Figure 2. The migration induced by NPY of VSMCs is attenuated by D_3 receptor activation. (A) Different concentration of D_3 receptor agonist (PD128907, 10^{-11}-10^{-8} M) had no effect on migration of VSMCs (n=5). (B) PD128907 dose-dependently (10^{-11}-10^{-8} M) attenuated the stimulatory effect of NPY (10^{-8} M) on VSMC migration. #P<0.05 vs. control; *P<0.05 vs. NPY (n=5). (C) The inhibitory effect of PD128907 on NPY (10^{-8} M)-mediated migration was significantly blocked by the D_3 receptor antagonist, U99194 (10^{-7} M). #P<0.05 vs. NPY; *P<0.05 vs. control (n=6). (D) The scratch assay indicated that the increased average migration distance, induced by NPY (10^{-8} M), was blocked by the D_3 receptor agonist, PD128907 (10^{-8} M), while in the presence of D_3 receptor antagonist U99194 (10^{-7} M), the effect of PD128907 was blocked. (E) Quantification of Fig. 2D, data are presented as the rate of VSMCs' average migration. #P<0.05 vs. NPY; *P<0.05 vs. control (n=6). Scale bars, 10 μm.The data are expressed as mean ± standard deviation. VSMCs, vascular smooth muscle cells; NPY, neuropeptide Y.

NPY-mediated VSMC migration is attenuated by PKA activation. The PKA inhibitor 14-22 (10^{-6}M) had no effect on VSMC migration or NPY-mediated VSMC migration, but blocked the inhibitory effect of PD128907 on VSMC migration, indicating that PKA was engaged in this process. Moreover, the inhibitory effect of PD128907 was imitated by the PKA activator, Sp-cAMP [S], in the presence of Sp-cAMP [S], the NPY-mediated stimulatory effect on VSMC migration was abolished (Fig. 3A). The authors initially determined the activity of PKA in VSMCs following treatment with PD128907 (10^{-8} M) or U99194 (10^{-7} M). It was identified that PD128907 increased PKA activity in VSMCs, while the D3 receptor antagonist, U99194 (10^{-7} M) attenuated PKA activity (Fig. 3B).

Discussion

NPY is widely distributed in the mammalian central nervous system and peripheral nervous system, together with catecholamine, which is released from the nervous endings, and serves an important role in the development of cardiovascular diseases, including atherosclerosis and hypertension. In the cardiovascular system, NPY is co-localized and co-released with norepinephrine, and they can be released under appropriate conditions of sympathetic nerve hyperactivity such as stress, hypertension and congestive heart failure (25-27). Previous studies demonstrated higher plasma NPY concentrations in hypertensive patients (28-30). Moreover, plasma NPY was elevated in hypertensive patients undergoing various

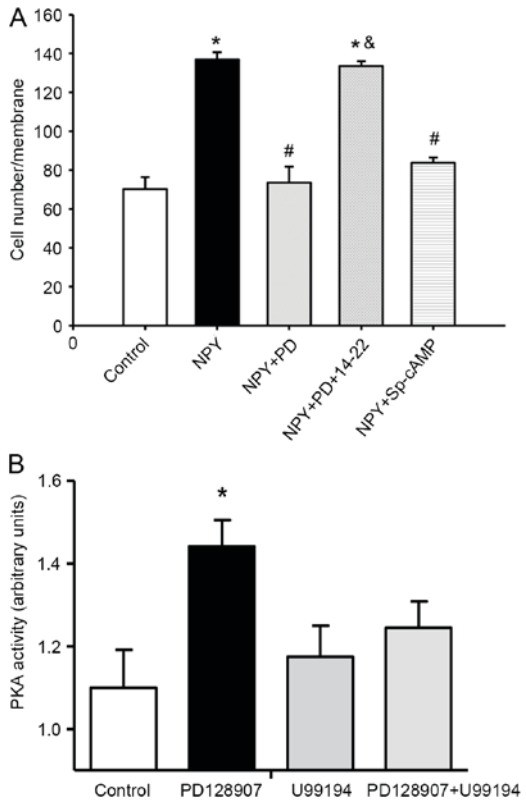

Figure 3. PKA activation significantly attenuated NPY-mediated migration of VSMCs. (A) VSMCs were treated with Sp-cAMP or NPY (10^{-8} M) with or without the presence of PKA inhibitor, VSMC migration was determined by Transwell migration assay. *P<0.05 vs. control; #P<0.05 vs. NPY; &P<0.05 vs. NPY+ PD128907 (n=5). (B) The activity of PKA under the treatment of PD128907 (10^{-8} M) or U99194 (10^{-7} M) was determined by cAMP-dependent protein kinase A assay. *P<0.05 vs. control (n=5). The data are expressed as mean ± standard deviation. NPY, neuropeptide Y; VSMCs, vascular smooth muscle cells; PKA, protein kinase A.

stress condition or exercise (31,32). These results suggested that endogenous NPY may serve an important role in pathogenesis of hypertension.

NPY receptors have five subtypes. Different NPY subtypes have been reported to be involved in different cardiovascular disease. For example, NPY Y_1 receptor is reported to engage in the NPY stimulation of DNA synthesis in human SMC grown from subcutaneous arteries and veins (33). In the rat model with balloon-injured artery, the expressions of NPY receptors, especially Y_1 and Y_5 receptor subtypes, were increased following injury (34-36). Similarly, increased NPY concentration is found in hypertension during pregnancy, a further study indicates that NPY, mainly via Y_5 receptor, induces VSMC proliferation and augments the increased blood pressure (37). Besides of hypertension, NPY and Y_1 receptors are involved in the mechanisms related to the development of atherosclerosis. $ApoE^{-/-}$ mice are intraperitoneally injected with two doses of NPY or Y_1 receptor antagonist. The Y_1 receptor antagonist increases atherosclerotic lesion areas in descending aortas in $ApoE^{-/-}$ mice (38). It is also demonstrated that cholesterol levels in serum is positively correlated with VCAM-1 expression and negatively correlated with NPY expression in aortic wall in mice treated with Y_1 receptor antagonist (39). Consistent with these reports, the present study found that NPY promoted VSMC migration in a dose-dependent manner; in the presence of Y_1 inhibitor, as presented in Fig. 1B, the effect of NPY was largely but not completely blocked, indicating that other NPY receptors may also contribute to the migration. In the VSMC, Y2 and Y5 also engaged in NPY-induced migration (10), but $Y_1$was the main vascular receptor (40,41).

Dopamine receptors are classified into two families: The D_1-like receptor subfamily includes the D_1 and the D_5 receptor, while the D_2, D_3, and D_4 receptors belong to the D_2-like subfamily. Both D_1 and D_3 receptors are expressed in VSMCs, and the activation of D_1 receptor relaxes blood vessels and decreases blood pressure. The D_3 receptor agonist, pramipexole decreases vascular resistance. Co-stimulation of D_1 and D_3 receptors has an additive vasorelaxant effect in the rat mesenteric artery when the mesenteric rings are pre-constricted with norepinephrine (42). This finding and other studies also demonstrated that activation of dopamine receptor inhibits some humoral factors-mediated VSMC proliferation. Stimulation of the D_3 receptor inhibited insulin receptor mRNA and protein expression and insulin-mediated VSMC proliferation (43). Dopamine D_1-like receptors suppress proliferation of vascular smooth muscle cell induced by insulin-like growth factor-1 (44). Besides VSMC proliferation in hypertension and atherosclerosis, VSMC migration also is important in the atherosclerotic formation. The present study suggested that activation of dopamine D_3 receptor inhibited NPY-mediated VSMC migration.

Numerous studies have reported that PKA is involved in the signaling pathway of dopamine receptors. Studies indicated that D_2-like dopamine receptors serve their physiological functions by regulating the PKA signaling pathway (45). In previous studies of the authors, the authors revealed that stimulation of D_3 receptors inhibits norepinephrine-and insulin-mediated VSMC proliferation via activation of PKA signaling (22,23). The current study stated that the D_3 receptor- mediated the inhibition of VSMC migration is also via the PKA pathway. The results of the present study indicated that PKA inhibitor 14-22 amide, the blocker of PKA, blocks the inhibitory effect of D_3 receptors on the VSMC migration is induced by NPY.

In conclusion, the dopamine D_3 receptor, via PKA, inhibits NPY Y_1 receptor-mediated migration of VSMCs, which may be involved in hypertension and atherosclerosis.

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