

ORIGINAL ARTICLE

# Inhibitory effect of D<sub>1</sub>-like dopamine receptors on neuropeptide Y-induced proliferation in vascular smooth muscle cells

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Proliferation of vascular smooth muscle cells (VSMCs) is thought to have a key role in the development of atherosclerotic lesions. Neuropeptide Y (NPY), norepinephrine and dopamine are sympathetic neurotransmitters. NPY has been particularly shown to stimulate proliferation of VSMCs. NPY, norepinephrine and dopamine are all sympathetic transmitters. In our previous study, we found that in the presence of the dopamine receptor, the  $\alpha_1$ -adrenergic receptor-mediated VSMC proliferation is reduced. We hypothesize that the activation of the D<sub>1</sub>-like receptor might inhibit the NPY-mediated VSMC proliferation. In our present study, we found that NPY, mainly via the Y<sub>1</sub> receptor, increased VSMC proliferation. This was determined by [<sup>3</sup>H]-thymidine incorporation, in a concentration ( $10^{-11}$  to  $10^{-8}$  M)-dependent manner. In the presence of the D<sub>1</sub>-like receptor agonist, fenoldopam ( $10^{-12}$  to  $10^{-5}$  M), the stimulatory effect of NPY on VSMC proliferation was reduced. The involvement of the D<sub>1</sub>-like receptor was confirmed when the inhibitory effect of fenoldopam was reversed in the presence of the D<sub>1</sub>-like receptor antagonist SCH-23390 ( $10^{-8}$  M). Moreover, the inhibitory effect of fenoldopam on NPY-mediated VSMC proliferation was also blocked in the presence of the PKA inhibitor 14–22 ( $10^{-6}$  M). Protein kinase A activator 8-(4-chlorophenylthio) adenosine-3,5-cyclic monophosphorothioate, Sp-isomer sodium salt ( $10^{-6}$  M) could simulate the stimulatory effect of fenoldopam. It indicated that the inhibitory effect of D<sub>1</sub>-like receptors on NPY-mediated VSMC proliferation may have an important role in the regulation of blood pressure or prevention of atherosclerosis.

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**Keywords:** atherosclerosis; neuropeptide Y; proliferation; vascular smooth muscle cells

## INTRODUCTION

Understanding the regulatory mechanism<sup>1–4</sup> of vascular smooth muscle cell (VSMC) proliferation is important in elucidating the pathogenesis of atherosclerosis as well as in the design of therapeutic drugs for the prevention of atherogenic disorders.<sup>5,6</sup>

Neuropeptide Y (NPY) is a 36-amino acid neurotransmitter that belongs to a family of peptides containing peptide YY and a pancreatic polypeptide. It was isolated originally from porcine brain and has been shown to be the most abundant neuropeptide in the mammalian central and peripheral nervous systems.<sup>7,8</sup> NPY, together with norepinephrine, ATP and dopamine, is released from the sympathetic nerve terminals to act as vaso-neurotransmitters. According to the duration of the vasoconstrictive activity, it is thought that ATP, norepinephrine and NPY mediate the immediate,<sup>9</sup> intermediate and long-lasting components of sympathetic nerve stimulation-induced vasoconstriction, respectively. Hypertension and atherosclerosis are chronic progressive diseases, therefore, the long-lasting effect, as mediated by NPY, seems to be much more important.

Dopamine has recently been characterized as an important modulator of blood pressure,<sup>10,11</sup> sodium balance and renal and adrenal functions, and it is relevant to the pathogenesis and/or maintenance of hypertension.<sup>12,13</sup> Dopamine receptors are classified into two families: the D<sub>1</sub>-like receptor subfamily includes the D<sub>1</sub> and the D<sub>5</sub> receptors, while the D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> receptors belong to the D<sub>2</sub>-like subfamily. Whereas the D<sub>1</sub>-like receptors couple to the stimulatory G protein, G $\alpha_s$ , D<sub>2</sub>-like receptors couple to the inhibitory G protein, G $\alpha_i$ /G $\alpha_o$ .<sup>13</sup> Our previous study showed that in the presence of the dopamine receptor, the  $\alpha_1$ -adrenergic receptor-mediated VSMC proliferation is reduced.<sup>4</sup> As a major mitogenic factor, NPY has been shown to stimulate the proliferation of VSMCs.<sup>3</sup> We hypothesize that the activation of the D<sub>1</sub>-like receptor might inhibit the NPY-mediated VSMC proliferation. Therefore, our present study was designed to investigate the possible role of D<sub>1</sub>-like receptors on NPY-mediated VSMC proliferation and examine its potential mechanism(s).

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## METHODS

### SD rat aortic smooth muscle cell culture

VSMCs were isolated from the aortic strips of male Sprague–Dawley (SD) rats (200–250 g) with an explant technique previously described.<sup>1,2</sup> Briefly, the arteries were dissected free of fat and excess adventitial tissue and then opened along its longitudinal axis, with the endothelial lining removed by vigorously scraping of the lumen with a scalpel blade. Small fragments (~1 mm<sup>3</sup>) were transferred to a flask and cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum supplemented with 1% penicillin/streptomycin/bFGF/insulin/EGF (all from Sigma, St Louis, MO) in a humidified atmosphere (95% air/5% CO<sub>2</sub>) 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  $\alpha$ -actin (Sigma). Cells from passages 3 through 5 were used. The cells were then placed in serum-free media for 24 h to render them quiescent.<sup>14,15</sup> The animal experiments were approved by the Animal Use and Care Committee of Daping Hospital.

### Measurement of [<sup>3</sup>H]-thymidine incorporation

Cell proliferation was determined by measuring the incorporation of [<sup>3</sup>H]-thymidine (Atomic Energy Research Establishment of China, Beijing, China) into the DNA of cells cultured in 96-well plates. After the induction of quiescence, VSMCs were re-stimulated with or without 10<sup>-8</sup> mol l<sup>-1</sup> NPY (Sigma) for 24 h. [<sup>3</sup>H]-thymidine (1  $\mu$ Ci ml<sup>-1</sup>) was added to each well 6 h before the measurements. At the end of the incubation period, the medium was removed and the cells were treated with 0.25 ml of 0.05% trypsin–0.53 mM EDTA for 5 min and diluted to 10 ml with ice-cold 1  $\times$  phosphate-buffered saline. The cells were incubated in ice-cold 10% trichloroacetic acid for 30 min. After being washed twice with phosphate-buffered saline, the cells were lysed with 0.2 M NaOH, neutralized with 0.2 M HCl and subjected to liquid scintillation counting (Beckman LS6500, Beckman, Fullerton, CA, USA). Three independent samples were measured in each experiment and the experiment was repeated at least three times.<sup>14,16</sup>

The D<sub>1</sub>-like receptor antagonists SCH-23390 (Sigma) was used to block the effect of the D<sub>1</sub>-like agonist, fenoldopam (Sigma). Owing to the fact that no ligand can distinguish between D<sub>1</sub> and D<sub>5</sub> receptors, the receptors involved in this experiment are likely to be D<sub>1</sub>-like receptors (D<sub>1</sub> and D<sub>5</sub>), which are expressed in VSMCs of rats. To determine whether the proliferative effect of NPY is exerted at the NPY Y<sub>1</sub> receptor, the NPY Y<sub>1</sub> receptor antagonist BIBP3226 (Sigma) was also used. A D<sub>3</sub> receptor agonist PD128907 (10<sup>-8</sup> M) and a D<sub>3</sub> receptor antagonist GR105691 (10<sup>-8</sup> M) were used for testing the role of D<sub>3</sub> receptor in NPY-induced VSMC proliferation.

### MTT assay

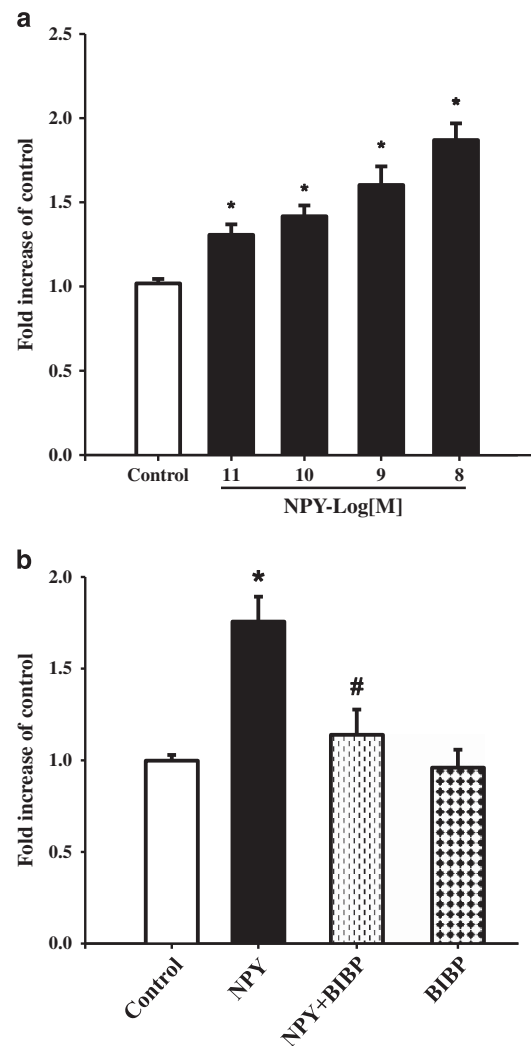
The viability of cells was determined on the basis of the activity of mitochondrial dehydrogenase to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) to formazan. Briefly, cells were plated in 96-well plates at a density of 1  $\times$  10<sup>3</sup> cells per well and treated with the indicated drugs for 24 h. MTT was then added to each well and incubation continued for 4 h in culture. Thereafter, 150  $\mu$ l of dimethyl sulfoxide was added to each well and an absorbance at 490 nm was read on a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).<sup>17,18</sup>

### Estimation of cell number

VSMCs were seeded in six-well plastic culture dishes at a density of 1  $\times$  10<sup>4</sup> cells per well in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and cultured for 24 h. After induction of quiescence, the culture media was replaced with serum-free media with the indicated drug and incubated for another 24 h. Cell viability was determined by the uptake of 0.4% Trypan blue after 5 min of mixing (Invitrogen Life Technologies, Carlsbad, CA), and were counted using a hemocytometer (Trypan blue uptake was observed in <10% of the cells). Counting was performed in triplicate.<sup>19</sup>

### Rat carotid artery balloon injury model

Carotid artery balloon injury was induced in male SD rats (230–300 g) as described in a previous study.<sup>20</sup> Briefly, rats were anesthetized using a mixture of ketamine (80 mg kg<sup>-1</sup>) and xylazine (12 mg kg<sup>-1</sup>), and then the left common and external carotid arteries were exposed through a midline cervical incision. A 2F Fogarty catheter (Edwards Life Sciences, Irvine, CA) was introduced into the common carotid artery through an arteriotomy in the external carotid artery and then the catheter was advanced to the proximal edge of the omohyoid muscle. To produce carotid artery injury, we inflated the balloon with saline and withdrew it three times from just under the proximal edge of the omohyoid muscle to the carotid bifurcation. After injury, the external carotid artery was ligated and the blood flow was restored.



**Figure 1** Effect of D<sub>1</sub>-like receptor on NPY-mediated proliferation of aortic vascular smooth muscle cells (VSMCs) from Sprague–Dawley (SD) rats. (a) Effect of NPY on VSMC proliferation. The primary cultured VSMCs were incubated with different concentration of NPY (10<sup>-11</sup> to 10<sup>-8</sup> M), the VSMC proliferation was determined by [<sup>3</sup>H]-thymidine incorporation. Results were expressed as fold increase of control. \**P*<0.05 vs. control (*n*=5). (b) Role of NPY Y<sub>1</sub> in the NPY-mediated VSMC proliferation. VSMCs were incubated with NPY (10<sup>-8</sup> M) in the presence of Y<sub>1</sub> receptor antagonist BIBP3226 (10<sup>-7</sup> M). The VSMC proliferation was determined by [<sup>3</sup>H]-thymidine incorporation. Results were expressed as fold increase of control. \**P*<0.05 vs. control, #*P*<0.05 vs. NPY (*n*=6). Analysis of variance with the Bonferroni *post hoc* test was performed to determine statistical significance. NPY, neuropeptide Y; BIBP, NPY Y<sub>1</sub> receptor antagonist BIBP3226.

After the balloon injury, rats were intraperitoneally injected with NPY (5 mg kg<sup>-1</sup> per day, i.p.) or similar volume of distilled water (2 ml kg<sup>-1</sup>) daily for 14 days. D<sub>1</sub>-like receptor activator fenoldopam (6 mg kg<sup>-1</sup> per day, i.p.) or D<sub>1</sub>-like receptor antagonist SCH-23390 (6 mg kg<sup>-1</sup> per day, i.p.) were administrated in different experimental groups. Then, both carotid arteries were dissected and the sections were stained with hematoxylin and eosin and observed under a light microscope (Olympus, Tokyo, Japan). Media and neointima areas of injured arteries were quantified by planimetry (μm<sup>2</sup>) and intima-to-media ratios were calculated as reported.<sup>21</sup>

### Immunohistochemistry staining

Briefly, paraformaldehyde-fixed vessel samples were conducted to paraffin-embedded sections (5 μm) and mounted on slides. The slides were incubated at 4 °C overnight, with or without primary anti Ki67 or a smooth muscle actin (α-SMA) antibodies at 1:1000 dilution. The slides were then incubated with secondary antibodies at 1:1000 at room temperature for 2 h. Pictures were taken under fluorescence microscopy. The antibodies were purchased from Invitrogen Life Technologies.

### Statistical analysis

The data are expressed as mean ± s.e.m. For analysis of differences among experimental groups, analysis of variance with the Bonferroni *post hoc* test was performed to determine statistical significance. A *P*-value of 0.05 was considered significant. In this study, *n* is the number of repeated sets of experiments for *in vitro* studies or the number of animals for *in vivo* studies.

## RESULTS

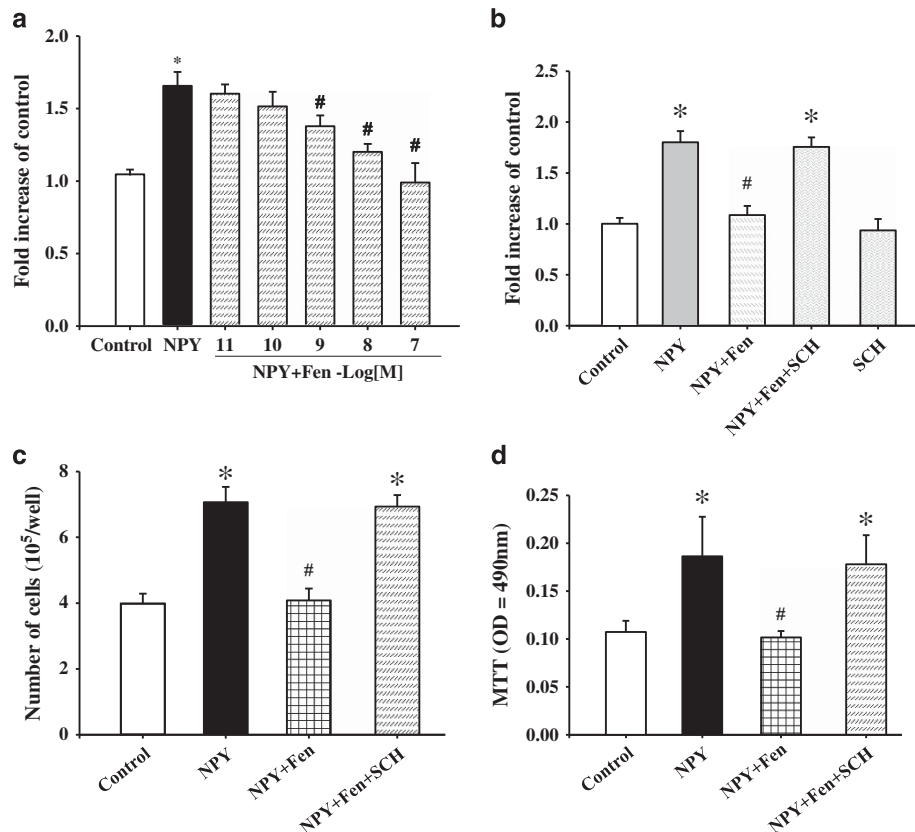
### NPY, via the NPY Y<sub>1</sub> receptor, increases proliferation of VSMCs from rat aorta of SD rats

Treatment of VSMCs with varying concentrations of NPY (10<sup>-11</sup> to 10<sup>-6</sup> M) for 24 h increased [<sup>3</sup>H]-thymidine incorporation in aortic VSMCs from SD rats in a concentration-dependent manner (Figure 1a). As major receptor subtypes of NPY receptors, we determined the role of Y<sub>1</sub> in the VSMC proliferation. Results showed that the increase in [<sup>3</sup>H]-thymidine incorporation induced by NPY was blocked by the NPY Y<sub>1</sub> receptor antagonist BIBP3226 (Figure 1b). This indicates that the NPY-mediated proliferation was via the Y<sub>1</sub> receptor.

### Effect of D<sub>1</sub>-like receptor on NPY-mediated proliferation of VSMCs

To investigate the interaction between NPY Y<sub>1</sub> and D<sub>1</sub>-like receptors, VSMCs were incubated with NPY and with the D<sub>1</sub>-like receptor agonist fenoldopam for 24 h. The results showed that fenoldopam reduced the stimulatory effect of NPY in a concentration-dependent manner (10<sup>-11</sup> to 10<sup>-7</sup> M; Figure 2a). The effect of fenoldopam was via the D<sub>1</sub>-like receptor, because the inhibitory effect of fenoldopam on NPY-mediated proliferation was blocked by the D<sub>1</sub>-like receptor antagonist SCH-23390 (Figure 2b).

To confirm the results from [<sup>3</sup>H]-thymidine incorporation study, we also determined the effect of fenoldopam on NPY-mediated VSMC proliferation, as indicated by cell number count and the MTT analysis. The data showed that NPY increased the VSMC number and MTT

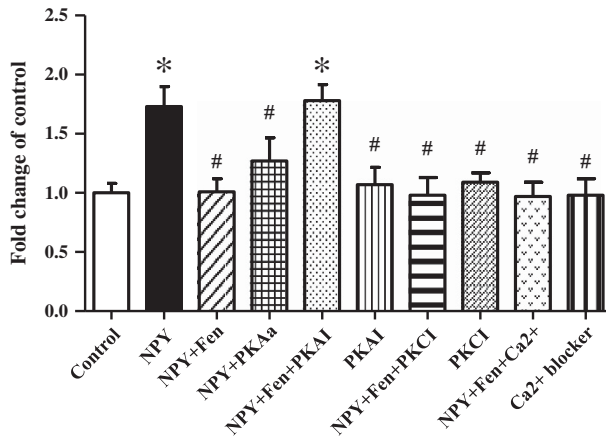


**Figure 2** Effect of D<sub>1</sub>-like receptors on neuropeptide Y (NPY)-mediated proliferation of vascular smooth muscle cells (VSMCs). (a) The dose-dependent effect of D<sub>1</sub>-like receptor agonist fenoldopam on NPY-mediated VSMC proliferation. The VSMC proliferation was determined by [<sup>3</sup>H]-thymidine incorporation. Results were expressed as fold increase of control (*n*=6). (b) Effect of a D<sub>1</sub>-like receptor antagonist SCH-23390 on NPY-mediated VSMC proliferation (*n*=7). (c and d) The VSMC proliferation was determined by cell number count (c) and MTT analysis (d) (*n*=5–6). \**P*<0.05 vs. control, #*P*<0.05 vs. NPY. Analysis of variance with the Bonferroni *post hoc* test was performed to determine statistical significance. Fen, fenoldopam; SCH, D<sub>1</sub>-like receptor antagonist SCH-23390 (10<sup>-8</sup> M).

viability, which were significantly reduced in the presence of the D<sub>1</sub>-like receptor agonist fenoldopam ( $10^{-7}$  M; Figures 2c and d).

### Role of PKA in the inhibitory effect by fenoldopam on VSMC proliferation

To explore the potential mechanisms for the effect of dopamine receptors on NPY-induced VSMC proliferation, we determined the



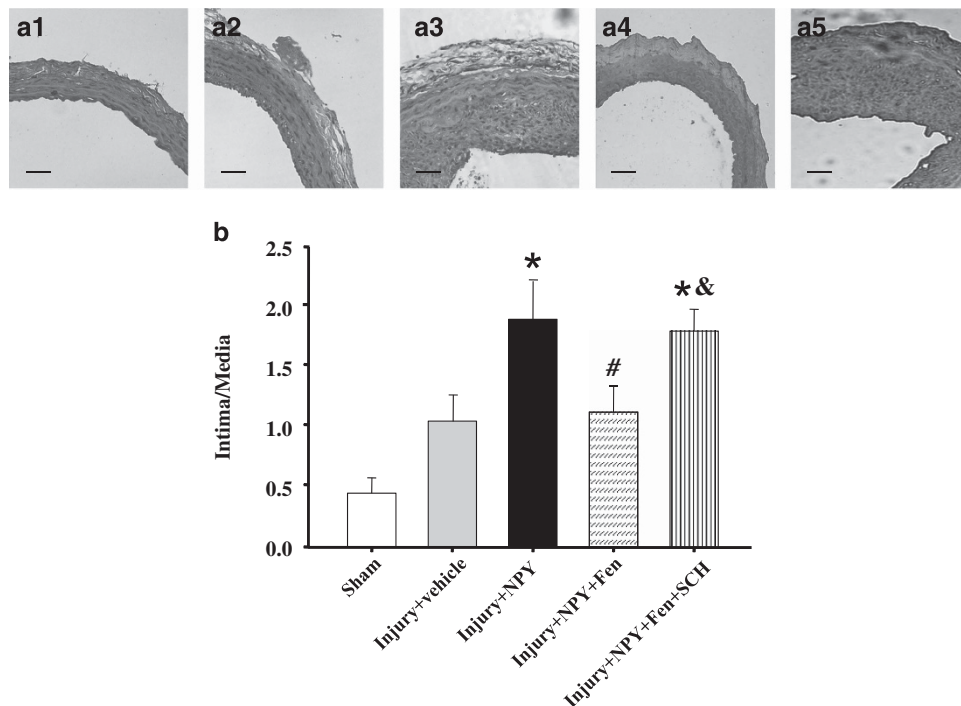
**Figure 3** Role of PKA and PKC signalings and Ca<sup>2+</sup> channel in the inhibitory effect of D<sub>1</sub>-like receptor on neuropeptide Y (NPY)-mediated vascular smooth muscle cell (VSMC) proliferation. VSMC proliferation was determined by [<sup>3</sup>H]-thymidine incorporation. Results were expressed as fold increase of control. \* $P < 0.05$  vs. control, # $P < 0.05$  vs. NPY ( $n = 6$ ). Analysis of variance with the Bonferroni *post hoc* test was performed to determine statistical significance. PKAa, PKA agonist Sp-cAMP[S] ( $10^{-6}$  M); PKAI, PKA inhibitor 14–22 amide ( $10^{-6}$  M).

roles of PKA and PKC signalings. Results showed that the PKA inhibitor (PKAI) 14–22 amide ( $10^{-6}$  M), by itself, had no effect on VSMC proliferation, but negated the inhibitory effect of fenoldopam, indicating that PKA was engaged in the inhibitory action of fenoldopam. To further confirm the role of PKA, we used the PKA agonist (PKAa), Sp-cAMP[S] ( $10^{-6}$  M) in this study. It showed that in the presence of Sp-cAMP[S], the stimulatory effect of NPY was reduced, which was blocked by PKA inhibitor 14–22 amide ( $10^{-6}$  M). PKA inhibitor itself did not influence VSMC proliferation (Figure 3). We also detected the role of PKC signaling and Ca<sup>2+</sup> channel in the antiproliferative effect of fenoldopam. The results showed that neither PKC inhibitor nor Ca<sup>2+</sup> channel blocker influenced the effect of fenoldopam on NPY-induced VSMC proliferation (Figure 3).

We have previously shown that PKA activation is the mechanism underlying the antiproliferative effects of D<sub>3</sub> receptor activation on norepinephrine-stimulated VSMCs.<sup>10</sup> We further determined the effects of D<sub>3</sub> receptor activation on NPY-stimulated VSMCs. As shown in Supplementary Figure 1, D<sub>3</sub> receptor activation with D<sub>3</sub> receptor agonist PD128907 blocked NPY-induced VSMC proliferation. The effect of D<sub>3</sub> receptor activation was blocked by a PKA inhibitor. These data indicated that the function of D<sub>3</sub> receptor activation is additive to the effect of D<sub>1</sub>-like receptor activation, which is associated with PKA signaling.

### Effect of D<sub>1</sub>-like receptor activation on angioplasty-induced neointima

To further identify the beneficial effect of D<sub>1</sub>-like receptor activation *in vivo*, we examined the role of fenoldopam in a rat model of atherosclerosis and neointimal formation according to the description in the previous study.<sup>20</sup> As shown in Figure 4, NPY significantly



**Figure 4** Effect of fenoldopam on angioplasty-induced neointima with neuropeptide Y (NPY) stimulation in a rat model. (a1–a5) Representative images of hematoxylin and eosin staining of rat carotid arteries from different treatment groups. (b) The bar graphs show quantification of the area of the intima-to-media ratio (I/M ratio).  $n = 5$ , \* $P < 0.05$  vs. injury+vehicle; # $P < 0.05$  vs. injury+NPY; & $P < 0.05$  vs. injury+NPY+Fen. Analysis of variance with the Bonferroni *post hoc* test was performed to determine statistical significance. Fen, fenoldopam. A full color version of this figure is available at *Hypertension Research* online.



promoted angioplasty-induced neointima, which was blocked by pretreatment with dopamine D<sub>1</sub>-like receptor activator fenoldopam. D<sub>1</sub>-like receptor antagonist SCH-23390 abolished the beneficial effect of fenoldopam. With Ki67 staining analysis, our data showed that NPY significantly promoted VSMC proliferation, which was blocked by fenoldopam. SCH-23390 abolished the antiproliferative effect of fenoldopam (Supplementary Figure 2). Thus, these data provided further evidence of the beneficial effect of D<sub>1</sub>-like receptor activation on atherosclerosis and neointimal formation.

## DISCUSSION

NPY has an important role in the regulation of blood pressure. It is reported that NPY accounts for about 30% of sympathetic nerve-mediated vasoconstriction in resistance vessels.<sup>22</sup> NPY potentiates other vasoconstrictor agents, such as norepinephrine, epinephrine and endothelin, to further increase blood pressure. Mice lacking the Y<sub>1</sub> receptor have a basal blood pressure, but lack the ability to potentiate norepinephrine-induced vasoconstriction. In addition, NPY has a hypertrophic effect on vascular smooth muscles,<sup>23,24</sup> as confirmed that NPY exerts direct stimulation to mRNA synthesis in VSMCs.<sup>2,25</sup> Furthermore, an NPY locus on chromosome 4 has been found to co-segregate with blood pressure in spontaneously hypertensive rats (SHRs). We also found that compared with Wistar–Kyoto rats, SHRs have higher plasma NPY concentration and higher NPY receptor-binding sites/affinity in VSMCs.<sup>25</sup> These evidences suggest that NPY is important in the development and maintenance of hypertension.

Previous studies have shown that dopamine receptors have an important role in the regulation of blood pressure.<sup>12,13</sup> Dopamine D<sub>1</sub>-like receptor-deficient mouse shows a higher blood pressure as compared with its control counterpart.<sup>25</sup> Stimulation of the D<sub>1</sub>-like receptor induces diuresis and natriuresis,<sup>12,26</sup> leading to vasodilation in arteries precontracted by other constrictive factors such as norepinephrine. VSMC proliferation has an important role in hypertension pathogenesis<sup>27</sup> and vascular remodeling in atherosclerosis.<sup>28,29</sup> In the hypertensive state, the D<sub>1</sub>-like receptor-mediated natriuresis is impaired due to receptor hyperphosphorylation,<sup>30</sup> which leads to its uncoupling from the G protein. As a major mitogenic factor, NPY has been shown to stimulate the proliferation of VSMCs.<sup>3</sup>

Our previous study found that in the presence of the dopamine receptor, the α<sub>1</sub>-adrenergic receptor-mediated VSMC proliferation is reduced. The present study also found D<sub>1</sub>-like receptor agonist reduced the NPY-mediated VSMC proliferation, indicating that the dopamine receptor inhibits the VSMC proliferation induced by NPY.

Dopamine D<sub>1</sub>-like receptor agonists increased PKA activity whereby reducing the MAPK activity in VSMCs, and it blocked the hypertrophic effect mediated by platelet-derived growth factor-BB in VSMCs.<sup>31</sup> In our present study, to determine the signal pathway involved in the inhibitory effect of fenoldopam, we observed the effect of different agonists/antagonists, including PKC agonist/antagonist, PKA agonist/antagonist and Ca<sup>2+</sup> channel blockers. The results showed that the inhibitory effect of fenoldopam is negated by PKA inhibition. Moreover, the PKA agonist could imitate the effect of fenoldopam, that is, PKA agonist blocks the effect of proliferation mediate by NPY in VSMCs.

The VSMC proliferation is a key process in the pathogenesis of atherosclerosis. VSMC proliferation is also associated with hypertension-related vascular dysfunction, restenosis and pulmonary hypertension.<sup>32</sup> NPY, released from the sympathetic nerve terminals as a vaso-neurotransmitter, acts via G protein-coupled receptors to stimulate constriction and VSMC proliferation through interactions with its Y<sub>1</sub> receptors.<sup>33</sup> NPY release is long-lastingly activated in these

pathological states, thereby inducing VSMC proliferation that contributes to the pathogenesis of these diseases. Our findings showed that the activation of the D<sub>1</sub>-like receptor inhibit the NPY-mediated VSMC proliferation. This may lead to developing new therapeutic strategies for the treatment of cardiovascular diseases related to VSMC proliferation.

In summary, we have demonstrated that the activation of D<sub>1</sub>-like receptor, via PKA signaling pathway, inhibits the proliferative effect of NPY in VSMCs. This indicates that the D<sub>1</sub>-like dopamine receptor may be a novel treating target for limiting the pathological progress of cardiovascular diseases such as atherosclerosis.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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Supplementary Information accompanies the paper on Hypertension Research website (<http://www.nature.com/hr>)