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Direct evidence of VEGF-mediated neuroregulation and afferent explanation of blood pressure dysregulation during angiogenic therapy

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Abstract

Objective: Oncocardiology is increasingly hot research field/topic in the clinical management of cancer with anti-angiogenic therapy of vascular endothelial growth factor (VEGF) that may cause cardiovascular toxicity, such as hypertension via vascular dysfunction and attenuation of eNOS/NO signaling in the baroreflex afferent pathway. The aim of the current study was to evaluate the potential roles of VEGF/VEGF receptors (VEGFRs) expressed in the baroreflex afferent pathway in autonomic control of blood pressure (BP) regulation. Methods: The distribution and expression of VEGF/VEGFRs were detected in the nodose ganglia (NG) and nucleus of tractus solitary (NTS) using immunostaining and molecular approaches. The direct role of VEGF was tested by NG microinjection under physiological and hypertensive conditions. Results: Immunostaining data showed that either VEGF or VEGFR2/VEGFR3 was clearly detected in the NG and NTS of adult male rats. Microinjection of VEGF directly into the NG reduced the mean blood pressure (MBP) dose-dependently, which was less dramatic in renovascular hypertension (RVH) rats, suggesting the VEGF-mediated depressor response by direct activation of the 1st-order baroreceptor neurons in the NG under both normal and disease conditions. Notably, this reduced depressor response in RVH rats was directly caused by the downregulation of VEGFR2, which compensated the up regulation of VEGF/VEGFR3 in the NG during the development of hypertension. Conclusion: It demonstrated for the first time that the BP-lowering property of VEGF/VEGFRs signaling via the activation of baroreflex afferent function may be a common target/pathway leading to BP dysregulation in anti-angiogenic therapy.

Keywords

autonomic control of blood pressure; cardiovascular toxicity; vascular endothelial growth factor; baroreflex; nodose ganglia; nucleus of tractus solitary

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1 Introduction

Vascular endothelial growth factor (VEGF) plays an important role in anti-angiogenic cancer therapy[1-3], but this clinical management can cause significant cardiovascular toxicity[4-6], such as hypertension[6-7] during the course of treatment. This observation has emerged as a hot topic in the field of oncocardiology which aims to retain the maximum therapeutic efficacy for cancers while minimizing adverse cardiovascular effects[8]. Yet, the cellular/molecular mechanism underlying the inhibition of VEGF/VEGF receptors (VEGFRs) creates a condition prone to hypertension thereby constituting an obstacle to the effective management of cardiovascular toxicity during anti-angiogenic cancer therapy. It has been well documented that VEGF/VEGFRs signaling plays a role in blood pressure (BP) reduction under both normotensive and hypertensive conditions[9-11] due at least partially to the nitric oxide (NO)-dependent pathway[11-13]. However, whether or not the autonomic control of circulation, especially the afferent loop of baroreflex[14-20], in cardiovascular dysregulation associated with VEGF/VEGFRs inhibition during cancer therapy has not yet been elucidated thus far. Additionally, epidemic evidence demonstrated that the incidence of hypertension[21-22] and risk for the development of certain tumors/cancers[23-24] are significantly higher in people who live under cold temperature. There is therefore a need for investigating the oncocardiology-based relationship between hypertension and anti-angiogenic therapy of cancer in the population residing in the north-east part of China because such a population is more vulnerable to cardiovascular disease or toxicity due to long-cold weather and the eating habits with high consumption of preserved foods containing high salt, high fat and high sugar to boost up energy against the cold weather in wintertime. The current study, therefore, aimed to elucidate the common pathway of baroreflex afferent loop for the cardiovascular toxicity/induced hypertension by blocking VEGF/VEGFRs signaling during angiogenic therapy, and to seek a potential target for clinical management of hypertension and pharmacological intervention that benefits people living in the cold region.

2 Methods

An expanded methods section is available in the online supplementary data.

2.1 Animals

Male Sprague Dawley (SD) rats (weighing 180-200 g) were purchased from the Experimental Animal Center of the Second Affiliated Hospital of the Harbin Medical University, and the animal certificate number was SCXK (Ha) 2006-010. Rats were provided with food and water ad libitum and were housed separately after surgery. The experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of the Harbin Medical University, which are in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and the National Institutes of Health publication “Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/readingroom/books/labrats/).”

2.2 Immunohistochemistry and whole section visualization

The protocol for NG immunohistochemistry was described in our previous report[16]. The preparation of brainstem sections (35 μm thicknesses, Bregma-12.6 mm) for immunostaining followed the procedures that were described in detail in our previous study[25].

2.3 Renovascular hypertension rat (RVH) models

Adult male SD rats were adaptively reared for 1 week in the animal facility after purchase. For preparation of RVH model, rats were anesthetized with 3% pentobarbital sodium (1.5 ml/kg, i.p.). A straight incision of about 2 cm was made on the left side of the spine at the lower edge of the twelfth rib. The subcutaneous tissue and muscle layer were carefully dissected to completely expose the left side kidney with renal artery that was subsequently narrowed with the silk thread. Each rat was then intramuscularly injected with 0.2 ml of penicillin per day for consecutive 3 days after surgery, and normal drinking water was supplied for 6 weeks. Systolic blood pressure (SBP) was monitored continuously by using tail-cuff method.

2.4 Nodose ganglion (NG) microinjection

In order to test the direct effect of VEGF on baroreflex afferent function, varying doses of VEGF or PBS as the vehicle control were microinjected directly into the NG (left side) using a precision glass micro-syringe (Hamilton, Reno, NE, USA) affixed with a 30G half-inch stainless steel syringe needle following the procedures described in our published articles[15-16].

2.5 Data analysis

Excel (v10, Microsoft) and Origin (v9 Microsoft) were used for data statistical analysis and graphical preparation. The two-tail Student t-test (paired and unpaired) and one-way ANOVA were applied where appropriate. The data are presented as mean ± SD unless otherwise specified and the P value less or equal to 0.05 was considered as significant difference.

3 Results

3.1 Distribution and localization of VEGF and VEGFRs in the NG and NTS

The baroreflex afferent loop is composed of baroreceptor terminals located at aortic arch and the cell body of baroreceptor neurons (1st-order) in the NG and baroreceptive neurons (2nd-order) in the NTS. To better understand the role of VEGF/VEGFRs signaling in the neurocontrol of BP regulation, it is essential to evaluate the distribution of either VEGF or VEGFRs in the NG and NTS, respectively. Upon reaching the cycle threshold (Ct) of qRT-PCR, the fluorescent signals against VEGF, VEGFR2, and VEGFR3 were obtained using immunohistochemical imaging. Clear fluorescence signals were detected in both HCN1-positive (presumably myelinated A-type afferents as pointed by yellow arrowhead) and HCN1-negative (presumably unmyelinated C-type afferents as indicated by white arrowhead) neurons of NG (Fig 1A). By comparison, the fluorescence signals for VEGF and VEGFR3 were mainly detected in the membrane of neurons, while that for VEGFR2 was only observed within the nucleus of NG neurons. These data suggest that VEGFR3 plays a more important role in VEGF-medicated baroreflex afferent function relative to VEGFR2. VEGF, VEGFR2 and VEGFR3 were also detected in the NTS region (Fig 1B).

Due to relatively higher Ct values and lower expression levels, immunostaining for VEGFR1 was not attempted in the NG and NTS. The negative control experiments were carried out for NG and NTS.

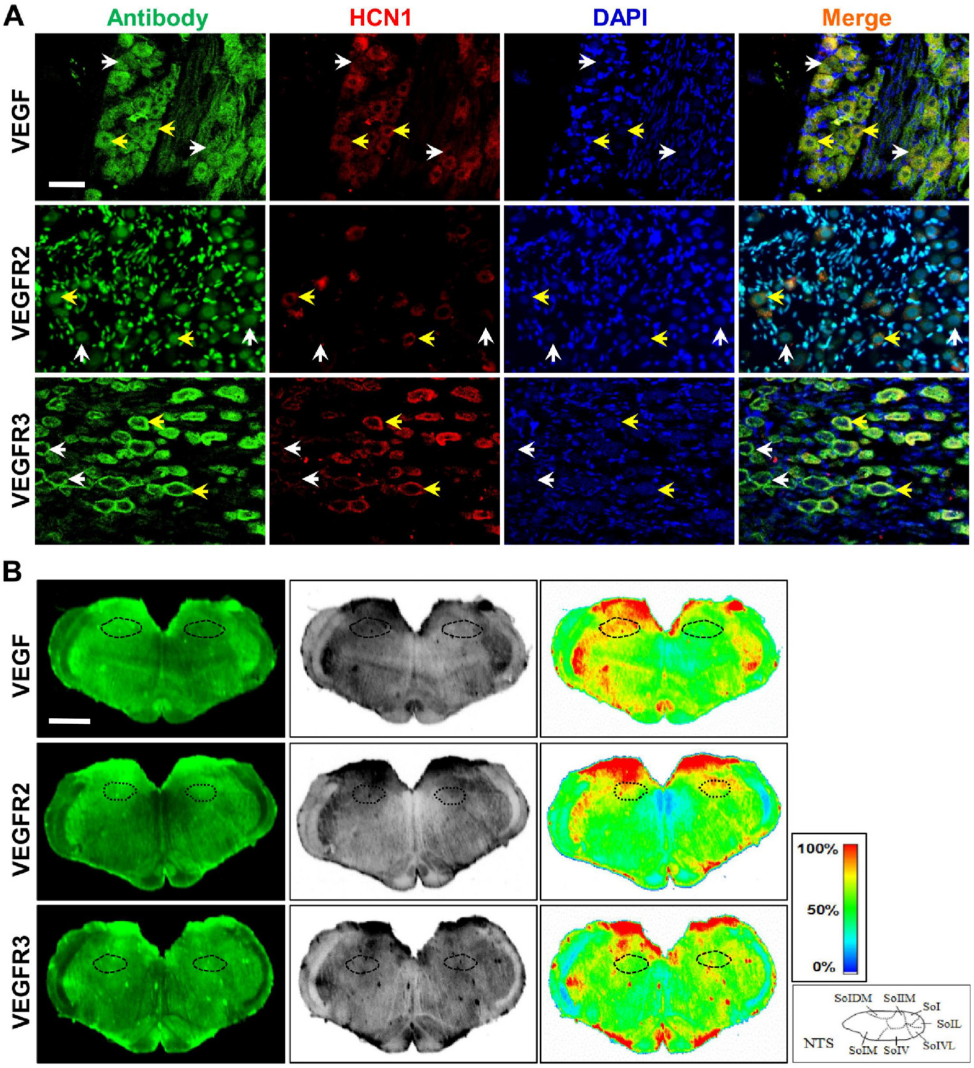


Fig. 1 Immunohistochemical characterization of the expression and distribution of VEGF, VEGFR2 and VEGFR3 in the NG and NTS. (A) The results of conventional immunohistochemical experiments carried out with specific antibodies against DAPI (blue), HCN1 (red), and VEGF, VEGFR2 or VEGFR3 (green) to detect the cellular/subcellular distributions of VEGF, VEGFR2 and VEGFR3 in the NG of adult male SD rats. Yellow and white arrowheads represent HCN1-positive (myelinated A-type afferents) and HCN1-negative (unmyelinated C-type afferents) neurons, respectively. Scale bar = 50 μm. (B) The representative immunostaining images of whole brainstem section showing the distribution of VEGF, VEGFR2 and VEGFR3 in the NTS. Sol: solitary tract; the scale bar = 2.0 mm. SolL: nucleus of the solitary tract (Sol); SolVL: -ventrolateral part; SolV: -ventral part; SolM: -medial part; SolDM: -dorsomedial part; SolIM: -intermediate part. The fluorescent bar represents the expression level. The negative staining as a control for both NG and NTS is presented in supplemental materials.

3.2 VEGF-mediated BP reduction in normotensive and hypertensive conditions

To test the direct activation of baroreceptor and consequent changes in BP reduction under normotensive or hypertensive condition, a rat model of renovascular hypertension (RVH) was successfully developed using adult male rats, as indicated by the significantly increased mean systolic BP from 115 mmHg (baseline) to 145 mmHg ($P < 0.05$ and $P < 0.01$ vs. shamed control, $n = 8$ for each group) from the 2nd week after the surgical procedures without significant changes in body weight (Fig 2A and Fig 2B). BP was reduced by direct microinjection of VEGF (Fig 2C) into the NG in a concentration-dependent manner in either control or RVH group ($n = 5$ for each group). It is worthy of mentioning that the degree of BP reduction was dramatically less in RVH than that in sham-control rats at 0.01 g/L and 0.03 g/L of VEGF ($P < 0.05$) (Fig 2D) and the reduced BP took a much shorter time ($P < 0.05$ or $P < 0.01$) back to control level (Fig 2E) at all tested concentrations of VEGF in RVH compared with sham-control rats. These results clearly indicate that direct activation of baroreceptor neurons by NG microinjection of VEGF leads to a depressor response under both physiological and hypertensive conditions and the less BP reduction observed in RVH rats is may be due at least in part to the impaired baroreflex sensitivity (BRS).

3.3 Impaired BRS in RVH rats

To test the direct effect of VEGF on BP reduction mediated through baroreflex, echocardiograph was performed to evaluate the alterations of cardiac function. The results showed that the averaged value of LVIDd was increased in RVH rats relative to sham-control counterparts ($P<0.05$, Fig 3A) without significant changes in other parameters, suggesting impaired diastolic cardiac function to compensate increased BP. Under such a condition, the impaired BRS might contribute to the less dramatic BP reduction in the present of VEGF following NG microinjection in RVH rats. In this regard, BRS was tested with serial concentrations of sodium nitroprusside (SNP) or epinephrine (PE), and the results showed that BRS was obviously reduced in RVH rats (Fig 3B-D) along with BP elevation (Fig 2A), suggesting that baroreflex afferent function is a key player in VEGF/VEGFRs-mediated autonomic control of BP regulation. A question yet to be addressed is if the observed changes in the expression of VEGF/VEGFRs are a molecular mechanism underlying the impaired baroreflex afferent function in the setting of renal hypertension.

3.4 Compensatory up-regulation of VEGF in response to VEGFR2 down-regulation in the NG with BP elevation in RVH rats

Based upon our in vivo tests with VEGF, the corresponding changes in the expression of VEGF and VEGFRs were expected to elevate BP in the NG and NTS of RVH rats. The BP-lowering property of VEGF/VEGFRs signaling was confirmed in our in vivo functional study (Fig 2), suggesting that the up-regulation of VEGF expression in the NG is a compensatory mechanism for the downregulation of VEGFRs. Notably, this notion is consistent with our results showing an up-regulation of VEGF along with downregulation of VEGFR2 at both mRNA and protein levels of NG (Fig 4A and Fig 4B). Generally, VEGFR2 plays a greater role in BP-lowering than VEGFR3, and thus simultaneously upregulated VEGFR3 in the NG (Fig 4C) could also be explained to compensate the downregulated VEGFR2 in RVH rats. Consistent with the findings obtained in the NG, similar results at both mRNA and protein levels were also observed in the NTS (Fig 4D-F).

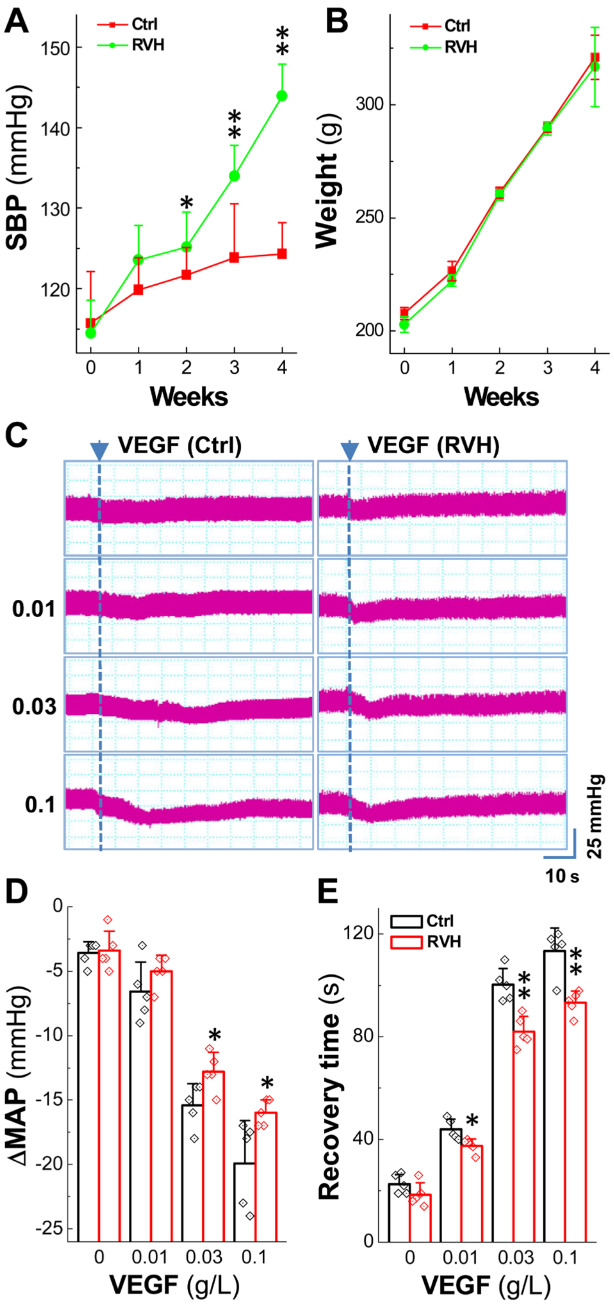
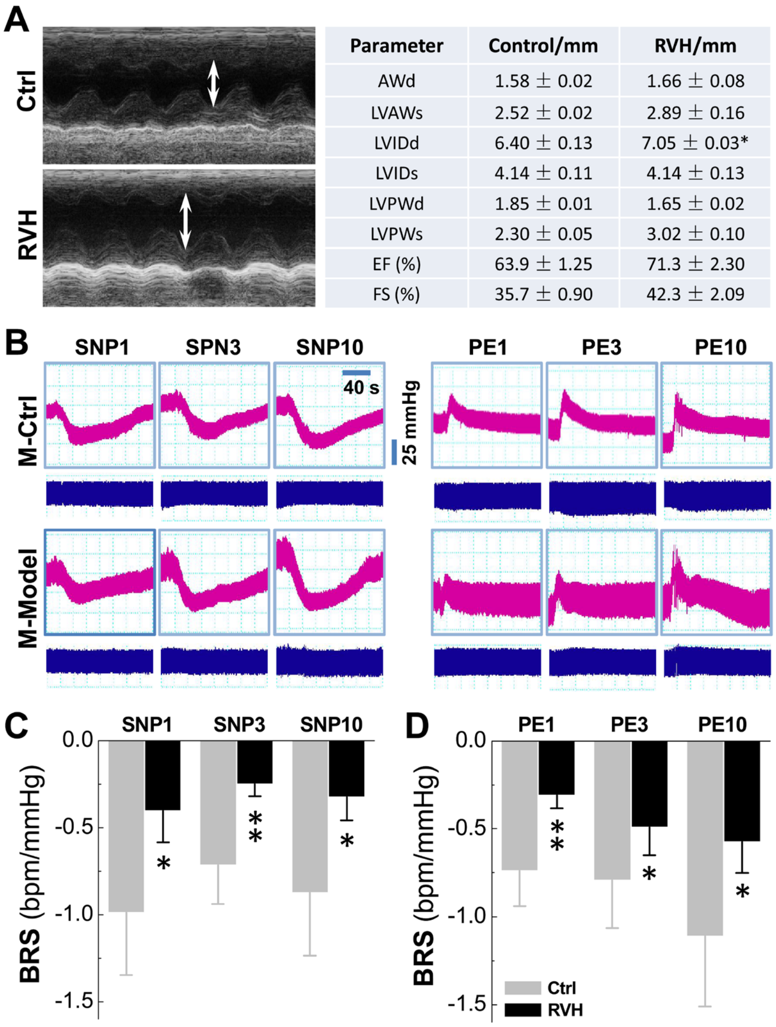


Fig. 2 VEGF-mediated BP reduction in control and RVH rats following direct microinjection of VEGF into the NG. (A and B) Changes in BP and body weight of Ctrl and RVH rats during 4 weeks after surgery; (C) Representative recordings of VEGF-medicated BP reduction following administration of a series of doses of VEGF via direct microinjection into the left side of the NG in control (Ctrl) and RVH model rats. Dotted lines represent the time points of microinjections; (D) Summarized data of VEGF-mediated BP reduction with different doses of VEGF; (E) Summarized data showing the recovery time of VEGF-mediated BP reduction. The data are expressed as mean ± SD and $n = 5$ rats for each group, $^*P < 0.05$ and $^{**}P < 0.01$ vs. Ctrl.



Parameter	Control/mm	RVH/mm
AWd	1.58 ± 0.02	1.66 ± 0.08
LVAWs	2.52 ± 0.02	2.89 ± 0.16
LVIDd	6.40 ± 0.13	7.05 ± 0.03*
LVIDs	4.14 ± 0.11	4.14 ± 0.13
LVPWd	1.85 ± 0.01	1.65 ± 0.02
LVPWs	2.30 ± 0.05	3.02 ± 0.10
EF (%)	63.9 ± 1.25	71.3 ± 2.30
FS (%)	35.7 ± 0.90	42.3 ± 2.09

Fig. 3 Changes in cardiac function, blood pressure, and baroreflex sensitivity in RVH rats. (A) Cardiac parameters determined by echocardiograph in control and RVH rats. Data are presented as mean ± SD, *n* = 4, *$P < 0.05$ vs. control (Ctrl). PSLAX: parasternal long axis; LVIDs/LVIDd: systolic/diastolic left ventricular internal diameter; IVSs/IVSd: systolic/diastolic left ventricular septum thickness; LVPWs/LVPWd: systolic/diastolic left ventricular posterior wall; EF: ejection fraction; FS: fractional shortening. (B) Representative recordings of mean arterial blood pressure (MAP, purple) and heart rate (blue) collected from Ctrl (*n* = 5) and RVH (*n* = 5) rats treated with 1, 3, and 10 μg/kg of SNP and PE, respectively. Scale bars are applied to all recordings. (C-D) The summarized changes of BRS (ΔHR/ΔMABP, bpm/mmHg) after treatment with SNP or PE at different concentrations. Data are expressed as means ± SD. *$P < 0.05$ and **$P < 0.01$ vs. Ctrl.

3.5 Expression alterations of VEGF/VEGFRs in the kidney of RVH rats

The kidney is the major place to produce VEGF in addition to the heart and brain, and it is therefore essential to monitor the expression of VEGF/VEGFRs at the gene and protein levels to further support our findings in the NG and NTS. The results from qRT-PCR and western blot analysis showed that VEGF expression was significantly reduced (Fig 5A) in RVH rats ($P < 0.05$ vs. Ctrl) due presumably to the ischemic injury of kidney induced by the surgical procedures for creating RVH, rather than to the up-regulation of VEGFR2 in response to the development of hypertension. However, the compensatory relationship (Fig 5B and Fig 5C) between down-regulated VEGFR2 and up-regulated VEGFR3 in RVH rats was tested only the tissue level of kidney in the present study.

4 Discussion

BP changes can be buffered by the baroreflex that starts from

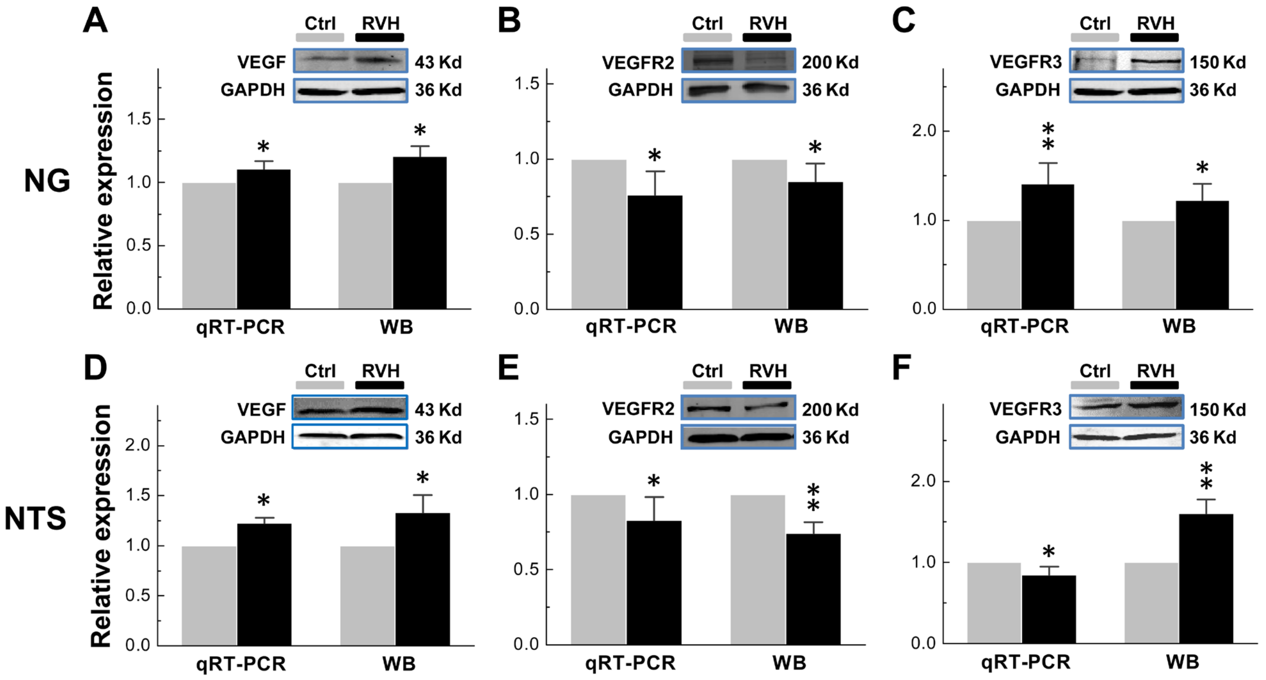


Fig. 4 Relative mRNA and protein expression levels of VEGF, VEGFR2 and VEGFR3 in the NG and NTS. (A-C) Relative mRNA and protein expression levels (fold change) and representative protein bends of VEGF, VEGFR2, and VEGFR3 in the NG of control and RVH model rats compared with internal control (GAPDH); (D-F) Relative mRNA and protein expression levels (fold change) and representative protein bends of VEGF, VEGFR2, and VEGFR3 in the NTS of control and RVH model rats compared with internal control (GAPDH). Data are presented as mean ± SD; *$P < 0.05$ and **$P < 0.01$ vs. Ctrl, $n = 4$ duplicated tests and tissue specimens collected from 6 rats for both PCR and Western blot analyses.

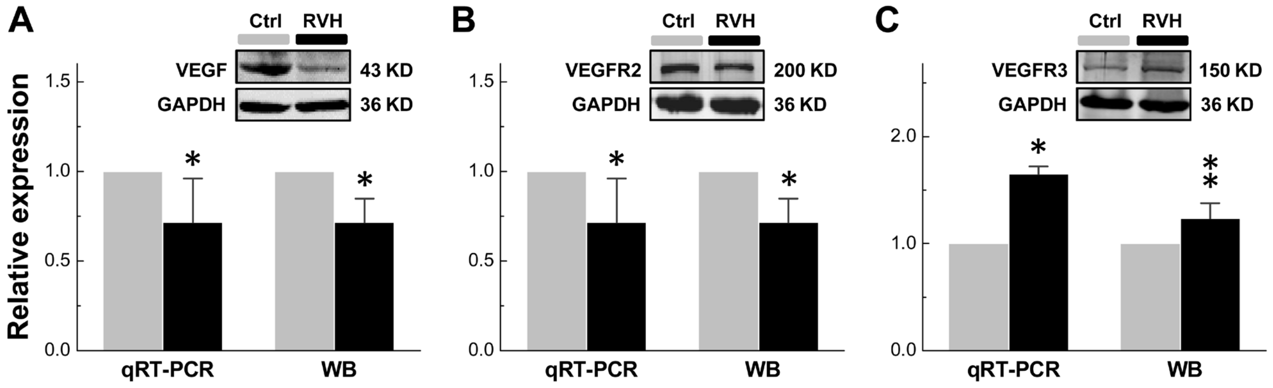


Fig. 5 The expression alterations of VEGF, VEGFR2 and VEGFR3 in the kidney. (A) Expression alterations (WB and PCR) of VEGF in the kidney of control and RVH rats compared with the internal control (GAPDH). Data are presented as mean ± SD; *$P < 0.05$ vs. control, $n = 4$; (B) Expression changes (WB and PCR) of VEGFR2 in the kidney of control and RVH rats compared with the internal control (GAPDH). Data are presented as mean ± SD; *$P < 0.05$ vs. control, $n = 4$; (C) Expression alterations (WB and PCR) of VEGFR3 in the kidney of control and RVH rats compared with the internal control (GAPDH). Data are presented as mean ± SD; *$P < 0.05$ vs. control, $n = 4$.

mechanosensitive channels (PIEZONs)[26] at baroreceptor terminals of aorta, the converted action potential discharge (bioelectric signals) from the blood pressure changes (mechanical signals) can be propagated to the 1st-order baroreceptor neurons with the cell bodies housed in the NG[27-28]. The signals are then relayed through their central projections to the medial NTS[29-31] of the dorsal medulla to form the synapse with the 2nd-order baroreceptive neurons. Baroreflex is the fastest among reflexes in mammals to stabilize the BP and heart rates along with changing the position, which remains plastic throughout life.

VEGF is the most important and specific angiogenic factor of a tumor or cancer, and excessive angiogenesis favors the development of cancers, suggesting that utilizing anti-angiogenic compounds could be a promising approach in tumor/cancer treatment. It is clear that cardiovascular toxicity, such as hypertension, is a great challenging for clinical management of cancer treatment during anti-angiogenic treatment against VEGF/VEGFR2 signaling and the mechanism underlying this cardiovascular side effect has not been fully elucidated. Even though the previous report has suggested that anti-angiogenesis-mediated hypertension is due likely to the dysregulation of nitric oxide (NO) production in circulatory system, the potential role of autonomic control of BP regulation, especially baroreflex afferent function, remained unstudied. Therefore, the present study aims to clarify the role and the underlying mechanism of the baroreflex afferent loop in regulating VEGF/VEGFRs signaling.

The baroreflex afferent loop is composed of baroreceptor terminals located at aortic arch and the cell body of baroreceptor neurons (1st-order) in the NG and baroreceptive neurons (2nd-order) in the NTS. A question remained unanswered is if VEGF and VEGFRs are expressed in the NG and NTS within the baroreflex afferent loop where the 1st-order and 2nd-order baroreceptive neurons are located, respectively. Our immunostaining and molecular observations confirmed the expression and distribution of VEGF and VEGFR2/R3 in the baroreflex afferent pathway under physiological conditions. Intriguingly, the expression of VEGFR2, a key player in BP-lowering, was significantly down-regulated in renovascular hypertension, which was accompanied by a compensatory up-regulation of VEGF and VEGFR3 presumably in response to higher BP and down-regulated VEGFR2. These results suggest that the BP-lowering effect of VEGF would be less dramatic in the renovascular hypertension mainly because of the downregulation of VEGFR2 in the afferent loop. Additionally, the direct depressor response to an increase in VEGF in the NG/NTS and weakened systemic BP drop due to a decrease in circulating VEGF released from injured kidney may be neutralized.

To mimic the direct effect on baroreceptor terminals and avoid the systemic BP drop due to impaired NO production, VEGF can be administered via microinjection directly into the NG, which can produce dose-dependent BP reduction under both physiological and renovascular hypertensive conditions. More importantly, BP reduction is significantly less dramatic in the hypertensive condition, which can be explained by a down-regulation of VEGFR2.

The finding (Fig 6) in the present study indicates that the baroreflex afferent pathway is likely a common target for the prevention and treatment of cardiovascular toxicity by blocking VEGF/VEGFRs signaling in cancer therapy. This finding also extends and improves our current understanding of the role of VEGF/VEGFRs in autonomic control of circulation and potential side effect of anti-angiogenic cancer therapy.

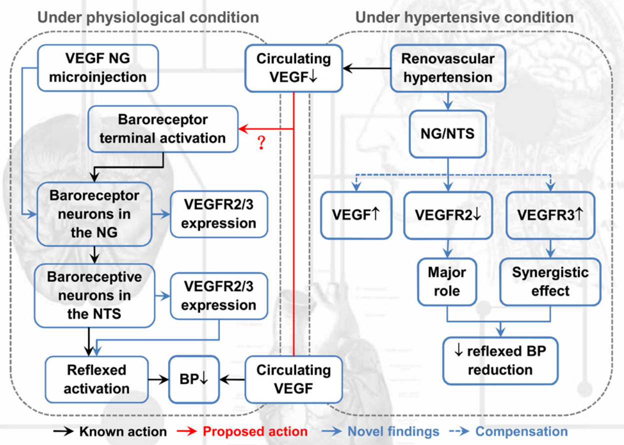


Fig. 6 Schematic diagram on the roles VEGF and VEGFRs signaling in neurocontrol of blood pressure regulation via the baroreflex afferent pathway, a common pathway of growth factor blockade-mediated cardiovascular dysregulation.

Ethical approval

All procedures performed in the animal studies were in accordance with the institutional guidelines for the care and use of animals declared in the methods part.

Conflicts of interest

These authors declared no conflict of interests.

Acknowledgments

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