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Mechanical stretch of sympathetic neurons induces VEGF expression via a NGF and CNTF signaling pathway

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ABSTRACT

Mechanical stretch has been shown to increase vascular endothelial growth factor (VEGF) expression in cultured myocytes. Sympathetic neurons (SN) also possess the ability to express and secrete VEGF, which is mediated by the NGF/TrkA signaling pathway. Recently, we demonstrated that SN respond to stretch with an upregulation of nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF). Whether stretch increases neuronal VEGF expression still remains to be clarified. Therefore, SN from the superior cervical ganglia of neonatal Sprague Dawley rats were exposed to a gradual increase of stretch from 3% up to 13% within 3 days (3%, 7% and 13%). Under these conditions, the expression and secretion of VEGF was analyzed. Mechanical stretch significantly increased VEGF mRNA and protein expression (mRNA: control = 1 vs. stretch = 3.1; $n = 3$/protein: control = 1 vs. stretch = 2.7; $n = 3$). ELISA experiments to asses VEGF content in the cell culture supernatant showed a time and dose dependency in VEGF increment due to stretch. NGF and CNTF neutralization decreased stretch-induced VEGF augmentation in a significant manner. This response was mediated in part by TrkA receptor activation. The stretch-induced VEGF upregulation was accompanied by an increase in HIF-1α expression. KDR levels remained unchanged under conditions of stretch, but showed a significant increase due to NGF neutralization. In summary, SN respond to stretch with an upregulation of VEGF, which is mediated by the NGF/CNTF and TrkA signaling pathway paralleled by HIF-1α expression. NGF signaling seems to play an important role in regulating neuronal KDR expression.

1. Introduction

Mechanical stretch has been shown to increase vascular endothelial growth factor (VEGF) expression in cultured myocytes [1]. Beside the biomechanical forces that occur in the developing or diseased heart, cells also from tissues such as lung, skeletal muscles and even cells within the scope of neoplastic genesis are subjected to biomechanical environments sufficient to produce stretches beyond the normal ranges [2–4]. In the heart, mechanical overload contributes to VEGF augmentation via a TGF-beta signaling pathway [5]. The heart is comprehensively innervated by the autonomic nervous system including sympathetic and parasympathetic nerve fibers controlling numerous cardiac regulating mechanisms. Therefore, the finding those sympathetic neurons (SN) possess the ability to produce and secrete angiogenic factors including VEGF is of obvious importance [6]. However, whether stretch also increases neuronal VEGF expression still remains to be clarified.

Beside its manifold effects on endothelial cells with the consequence of forming new collaterals VEGF also has direct multiple effects on SN [7]. For instance, in superior cervical ganglia (SCG) of newborn rats, nerve growth factor (NGF) induces capillary sprouting via the release of VEGF [8]. The NGF induced VEGF upregulation in SN is mediated by TrkA receptor activation [9]. Taken together, these findings indicate that neuronal cells may regulate their own VEGF requirements depending on the preexisting biomechanical forces in an autocrine/paracrine fashion by a NGF and TrkA signaling pathway.

Abbreviations: NGF, nerve growth factor; NT-3, neurotrophin-3; GDNF, glial cell-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; TrkA, tyrosine kinase receptor A; VEGF, vascular endothelial growth factor; KDR, VEGF Receptor 2; HIF-1α, hypoxia-inducible factor-1 alpha; SCG, superior cervical ganglia; SN, sympathetic neurons; MI, myocardial infraction.

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Likewise, NGF has been shown to promote angiogenesis in ischemic hind limbs [10] and to recover cardiac function after myocardial infarction (MI) [11]. During MI intracardiac cells are exposed to increasing stretch. Stretch is one important contributor to neuronal NGF and CNTF expression [12], whereas cardiomyocytes respond to stretch with a down-regulation of NGF [13]. Up to now, it is quite uncertain if the beneficial effects observed by NGF signaling in terms of increasing wall stress (e.g. MI) are due to a direct NGF function or whether stretch-induced NGF and CNTF secretion promotes neuronal VEGF expression in an autocrine/paracrine fashion subsequently contributing to neovascularisation and recovering heart function.

We hypothesized that SN respond to stretch with an up-regulation of VEGF and that neuronal NGF and CNTF secretion may be involved in this signaling pathway. Therefore, we used an in vitro model of cultured SN from the SCG of neonatal rats and analyzed the expression of VEGF under conditions of increasing stretch over a time period of 3 days.

2. Materials and methods

2.1. Cell cultures of superior cervical ganglia (SCG) and application of mechanical stretch

All animal experiments were approved by the local Ethics in Animal Research Committee, University RWTH Aachen, Germany (TV-No.: 10596 A). The investigation also conforms to the Guide for Care and Use of Laboratory Animals US (NIH Publication No. 85-23, revised 1996). Primary cultures of SCGs were performed from postnatal day 1–3 Sprague Dawley rats (Charles River, Germany) as described previously [12]. Cells were incubated on six well dishes with flexible membranes (Bioflex Collagen Plate I, Flexcell) at a density of 100.000 cells per well. "*n*" represents the number of cell preparations. For each SCG cell preparation we used in average 25–30 neonatal rats. During the first 48 h SCG neurons were grown in serum-containing medium. Since myocardial dilatation generally develops in a chronic scenario, SN were exposed to a gradual increase of stretch from 3% up to 13% within 3 days (3%, 7% and 13%) in a complete serum free medium [12–14]. Un-stretched control cells were treated equally without application of mechanical stretch.

2.2. RNA preparation, first-strand cDNA synthesis and quantitative real-time PCR

RNA extraction, first-strand cDNA synthesis and quantitative real-time PCR experiments were performed as described previously [15]. PCR primers and fluorogenic probes for target genes and the endogenous control were purchased from Applied Biosystems (Foster City, CA). The assay numbers were as follows: Rn00560865_m1 (beta-2 microglobulin), Rn01511602_m1 (VEGF).

2.3. ELISA for VEGF

For VEGF ELISA the conditioned medium was collected before initiation of stretch as well 24, 48 and 72 h after exposure to mechanical stretch. Samples were assayed using a VEGF kit from R&D Systems (Minneapolis, USA) according to manufacturer's instructions. Data were collected at a wavelength of 450 nm on a microplate reader (Spectrafluor Plus from Tecan).

2.4. Western blot

Western blotting with equal amounts of proteins was performed as described previously [16].

2.5. Antibodies and neurotrophin neutralizing antibodies

Following primary antibodies were used: rabbit anti-VEGF-A (Acris Antibodies), rabbit anti-KDR (Santa-Cruz, sc-6251) and rabbit anti-GAPDH (Cell Signaling, #2118). Neurotrophin neutralizing antibodies: anti-NGF (Sigma–Aldrich, #N6655, 1:500 dilution), anti-CNTF (R&D Systems, #MAB557, 5 μg/ml), anti-GDNF (R&D Systems, #MAB212, 5 μg/ml), anti-NT-3 (Promega, #G1651, 5 μg/ml), HIF-1α (Santa Cruz, sc-12542). Receptor blockers: anti-TrkA (R&D Systems).

2.6. Viability measurements with human coronary artery endothelial cells (HCAEC)

To examine the biological activity of neuronal VEGF secreted under conditions of mechanical stretch we cultivated HCAEC (Promocell, Germany) and performed the WST-8 colorimetric Assay kit (Promocell, Germany) according to the manufacturer's instructions. HCAEC were incubated in 96 well plates at a density of 3×10^4 cells/well for 48 h with cell conditioned medium of SN. Controls of HCAEC were incubated only with endothelial cell growth medium (Promocell, Germany) for 48 h. The viability experiments were repeated with the cell conditioned medium from two different SN cell preparations. Each condition included measurements of 16 wells.

2.7. Statistical analysis

All values are expressed as mean ± SEM. Comparisons of two groups were made by the student's *t*-test and multiple groups were made by 1-way ANOVA followed by Bonferroni's post hoc test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Mechanical stretch increases neuronal VEGF expression

SN were exposed to a gradual increase of stretch as described in the method section. Stretch over a time period of 72 h resulted in a significant increase in VEGF gene expression (Fig. 1A). The higher VEGF mRNA content was paralleled by an increase in VEGF protein expression as confirmed by Western blotting (Fig. 1B). Morphological nerve sprouting due to stretch by 13% could be detected by microscopical images as shown in Fig. 1C.

3.2. The role of NGF and CNTF in stretch-induced neuronal VEGF expression

Recently, we demonstrated that mechanical stretch of SN resulted in nerve sprouting mediated by an increase in neuronal NGF and CNTF expression [12]. To further characterize the role of NGF and CNTF in stretch-induced VEGF expression we performed loss of function experiments with NGF or CNTF neutralizing antibodies. We found that the stretch-induced neuronal VEGF expression returned almost to baseline levels in the presence of NGF or CNTF neutralizing antibodies (Fig. 2A and B). CNTF neutralization showed a tendency to be more effective in preventing stretch-induced VEGF expression as compared to NGF neutralization, but statistically we observed no significant differences between NGF and CNTF neutralization in our cell culture model. Using highly sensitive VEGF ELISA kits we further analyzed the secretion of VEGF in the cell culture supernatant of SN. These experiments revealed a time and dose dependency of stretch-induced neuronal VEGF expression. During the first 24 h of stretch by 3%, VEGF amounts remained on baseline levels (data not shown). From 24

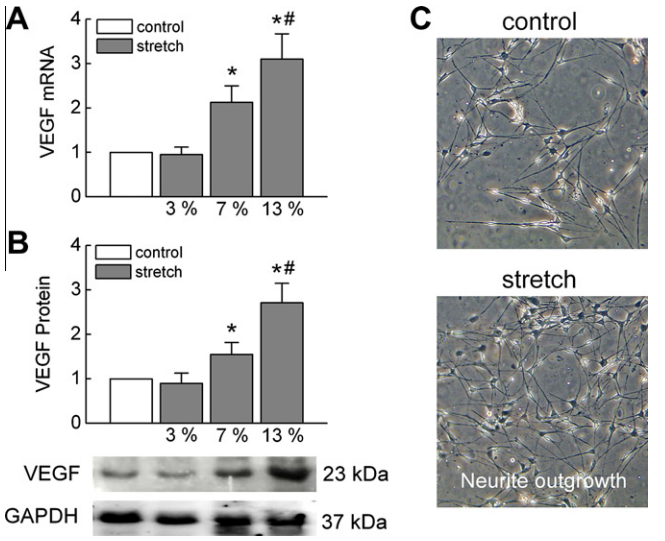

Fig. 1. Mechanical stretch of SN increases VEGF mRNA and protein expression in a time and dose depended manner. SN were exposed to a gradual increase of mechanical stretch over a time period of 72 h. (A) Real-time PCR showed a time and dose depended increase in VEGF mRNA expression by stretch up to 13%. (B) The increase in mRNA expression was paralleled by an almost 2,5-fold increase in VEGF protein expression after 72 h of stretch by 13%. (C) Microscopic images showing control and stretched neurons in cell culture. All PCR and WB data are derived from at least $n = 3$ cell preparations, $^{*}p < 0.05$ vs. control cells. $^{\#}p < 0.05$ vs. stretch by 7%.

to 48 h of stretch by 7% we observed a slight but insignificant increase in VEGF expression, which became highly significant after 72 h of stretch by 13% (Fig. 2C and D). NGF or CNTF neutralization could prevent this stretch-induced VEGF increase in a significant manner (Fig. 2C and D).

3.3. Neurotrophin-3. (NT-3) or glial cell-derived neurotrophic factor (GDNF) loss of function experiments found no significant effect on stretch-induced neuronal VEGF induction

To analyze whether neurotrophic factors other than NGF or CNTF play a role in neuronal VEGF induction, we extended the loss of function experiments by NT-3 or GDNF neutralization. We found that neither NT-3 nor GDNF neutralization significantly altered stretch-induced VEGF expression in our neuronal cell cultures (Fig. 3A and B).

3.4. Stretch-induced neuronal VEGF secretion is biologically active

To investigate whether neuronal VEGF secreted due to stretch shows a biological activity, we performed cell viability experiments with HCAEC as described in the method section. Conditioned medium from stretched neurons showed significant higher cell viability levels in HCAEC as compared to HCAEC incubated with conditioned medium from neurons not exposed to stretch (Fig. 3C). Whereas NGF or CNTF neutralization showed a preventive effect on HCAEC proliferation, NT-3 or GDNF neutralization showed a slight tendency that was statistically not significant.

3.5. Stretch-induced neuronal VEGF expression is mediated in part by TrkA signaling

TrkA activation by NGF signaling has been reported to mediate VEGF augmentation in SN [9]. Our results confirm these findings in a different setup. As an ubiquitous stimulus in the developing and diseased heart, stretch contributes to VEGF induction in SN via TrkA receptor downstream signaling (Fig. 3D). In our cell culture model of stretched neurons, this signaling route seems to be only a part of the puzzle, since the VEGF mRNA increase induced by stretch did not completely return to baseline levels by TrkA

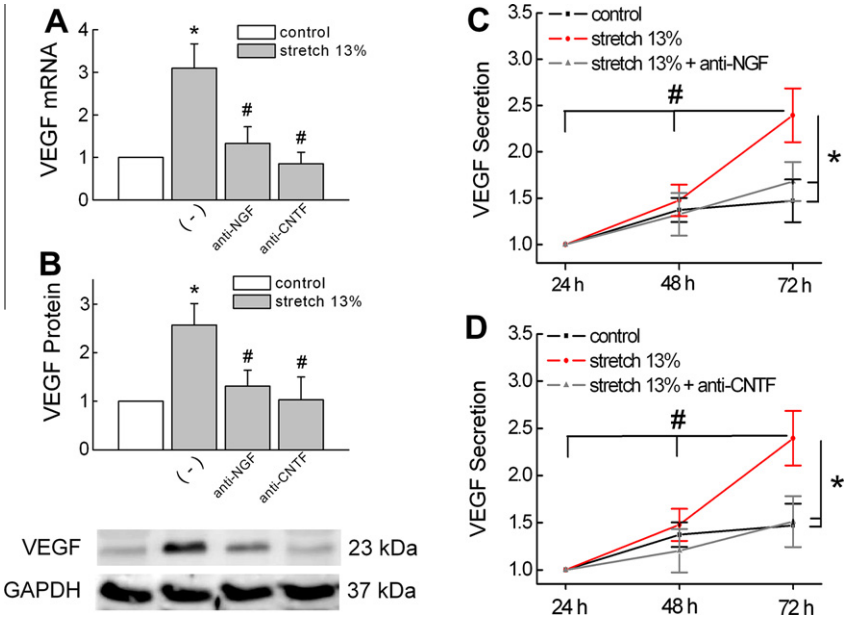

Fig. 2. Stretch-induced neuronal VEGF augmentation is mediated by NGF or CNTF. SN were exposed to a gradual increase of mechanical stretch over a time period of 72 h in the presence or absence of NGF or CNTF neutralizing antibodies. (A) On mRNA level NGF or CNTF neutralization blunted the stretch-induced VEGF increase almost to control levels. (B) WB experiments could confirm these findings on protein level. (C-D) ELISA experiments confirmed the time and dose dependency of stretch-induced VEGF augmentation. For VEGF ELISA the conditioned media was collected at 24, 48 and 72 h after exposure to mechanical stretch and samples were assayed by a VEGF kit from R&D Systems (Minneapolis, USA) according to manufacturer's instructions. (A-B) VEGF expression increased significantly at 72 h by 13% of mechanical stretch as compared to 24 and 48 h of control, stretched cells and stretched cells in the presence of neutralizing antibodies as well to 72 h of control cells and stretched cells in the presence of NGF or CNTF neutralizing antibodies. All PCR, WB and ELISA data are derived from at least $n = 3$ cell preparations. (PCR and WB) $^{*}p < 0.05$ vs. control cells. $^{\#}p < 0.05$ vs. stretch by 13%. (ELISA) $^{*}p < 0.05$ vs. 72 h of control or stretched cells in the presence of NGF or CNTF neutralizing antibodies. $^{\#}p < 0.05$ vs. 24 and 48 h of control, stretched cells and stretched cells in the presence of NGF or CNTF neutralizing antibodies.

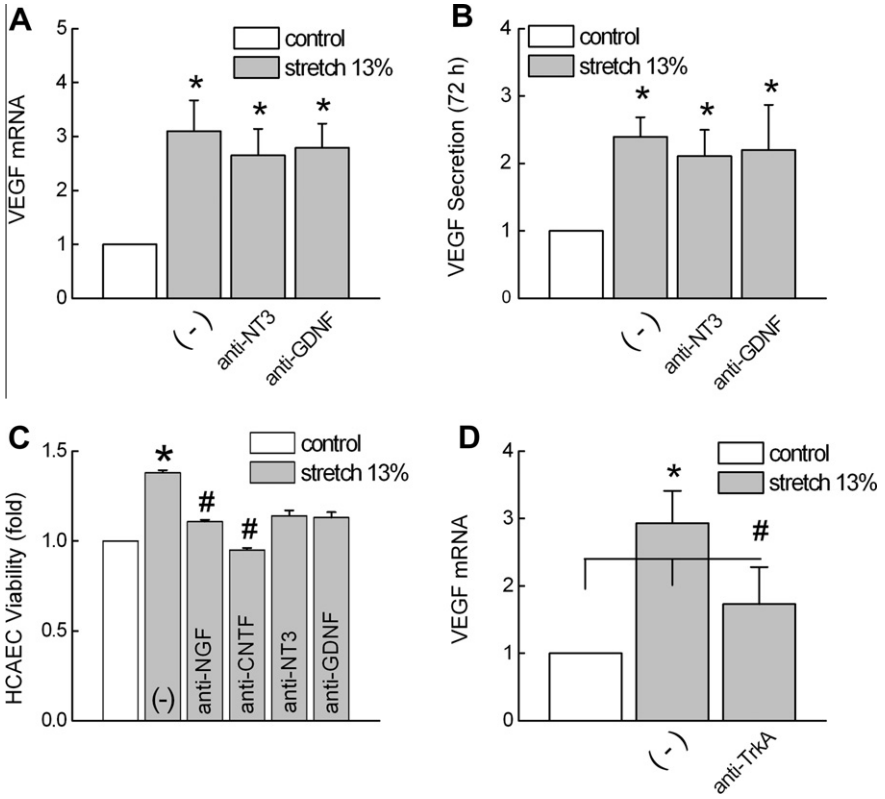

Fig. 3. NT-3 or GDNF remained without significant effect on stretch-induced neuronal VEGF expression. (A-B) ELISA data are presented in panel diagrams with samples from 72 h. Neither NT-3 nor GDNF could prevent the stretch-induced VEGF increase on mRNA or protein level. (C) To demonstrate the biological activity of neuronal VEGF secreted in terms of mechanical stretch, endothelial cells from human coronary arteries (HCAEC) were incubated with SN cell-conditioned medium for 48 h. HCAEC viability experiments showed that only NGF or CNTF neutralization was able to block endothelial cell proliferation in a significant manner, whereas NT-3 or GDNF cell conditioned medium of SN showed a slight tendency, but this observation was statistically not significant. (D) TrkA receptor blockade was capable to prevent stretch induced neuronal VEGF augmentation. This response was statistically significant, but failed to blunt the stretch effect completely. All ELISA data are derived from $n = 3$ cell preparations and were repeated twice. All PCR data are derived from at least $n = 3$ cell preparations. $^{*}p < 0.05$ vs. control cells. $^{\#}p < 0.05$ vs. stretch by 13%.

blockade, whereas NGF or CNTF neutralization was effective in leading neuronal VEGF mRNA levels to values within the baseline range. Further studies are required to reveal the underlying mechanisms by which stretch affects neuronal VEGF induction.

3.6. Neuronal HIF-1α is upregulated by mechanical stretch

Next, we investigated whether mechanical stretch of SN is accompanied by HIF-1α upregulation. Stretch by 13% contributed to a significant increase in neuronal HIF-1α expression (Fig. 4A). Our results confirm previous work that has shown that HIF-1α is involved in neuronal VEGF induction, which is mediated by NGF and TrkA signaling [9].

3.7. Neuronal KDR expression depends on NGF signaling

In the next step we investigated whether stretch affects neuronal KDR expression. We found that stretch remained without a significant effect on neuronal KDR expression compared to unstretched control cells, whereas NGF neutralization showed a marked effect on neuronal KDR expression. NT-3, CNTF or GDNF neutralization remained without significant effects (Fig. 4B).

4. Discussion

The major findings of the present study are: (a) SN respond to stretch with an increase in VEGF expression and secretion. (b) Neuronal VEGF augmentation due to stretch shows a time and dose dependency. (c) NGF and CNTF are important contributors to stretch-induced neuronal VEGF induction. (d) The stretch-induced neuronal VEGF induction is mediated in part by TrkA activation and is accompanied by HIF-1α upregulation. (e) NGF signaling seems to play an important role in regulating neuronal KDR expression.

4.1. Mechanical stretch and biochemical answers in the cardiovascular system

Stretch occurs in the cardiovascular system in different forms and degrees (cyclic stretch, static baseline stretch and stretch due to increasing shear forces). During MI or arterial hypertension intracardiac cells are exposed to increasing baseline stretch. Regarding neurotrophic regulation in terms of stretch, cardiomyocytes decrease NGF expression whereas SN respond to stretch with an upregulation of various neurotrophins including NGF and CNTF [12,13]. The balance between these two cellular systems still remains to be elucidated. In the present study we showed that stretch of SN goes along with VEGF induction in a time and dose dependent fashion. NGF and CNTF signaling seem to play a considerable role in this scenario, whereas a significant influence of NT-3 or GDNF on neuronal VEGF induction could be ruled out. In addition, neuronal VEGF levels secreted under conditions of mechanical stretch showed a biological activity as confirmed by HCAEC viability experiments.

4.2. VEGF expression in cardiomyocytes and SN

VEGF is expressed in the heart and its expression is markedly increased in response to hypoxia and mechanical overload

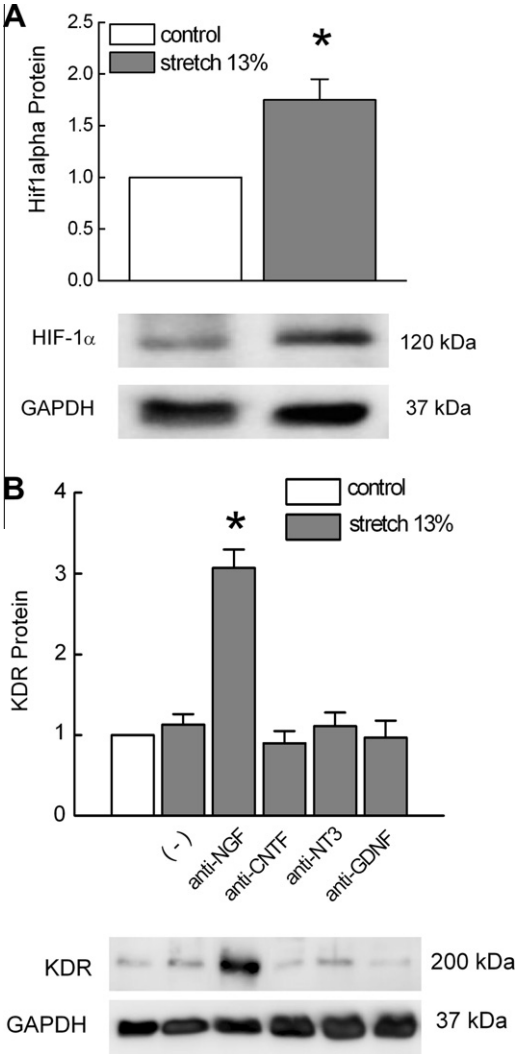

Fig. 4. The role of stretch and NGF signaling on neuronal Hif-1 α and KDR expression. (A) Stretch by 13% contributed to an increase in neuronal Hif-1α expression, (B) but remained without a significant effect on neuronal KDR expression. NGF neutralization increased neuronal KDR expression significantly, whereas CNTF, NT-3 or GDNF neutralization remained without effect. All WB data are derived from at least $n = 3$ cell preparations, $^{*}p < 0.05$ vs. control cells.

[17,23,24]. As one cellular source, the single cardiomyocyte responds to stretch with an upregulation of VEGF [1]. Since the autonomic nervous system of the heart plays an essential role in the regulation of diverse physiological and pathological phenomena, the knowledge that SN also possess the ability to produce and secrete VEGF is of obvious importance [6]. As shown by other groups in a different experimental setup, neuronal VEGF expression depends on autocrine/paracrine NGF and TrkA signaling [9]. Our results could confirm and extend these findings in a different cell culture scenario. In our in vitro model of cultured SN, we found that neuronal VEGF induction due to a gradual increase of baseline stretch was mediated in part by a TrkA signaling pathway. TrkA inhibition significantly decreased stretch-induced VEGF mRNA expression, but was not effective in preventing it completely. These data show that TrkA signaling plays a considerable role in regulating neuronal VEGF expression in terms of mechanical stretch.

4.3. The autonomic nervous system and the role of VEGF

The sympathetic nervous system mediates its regulatory mechanisms on the cardiovascular system via postganglionic sympathetic neurons, which are innervating blood vessels and the heart. The vascular sympathetic innervation of vessels has a major impact on blood pressure and blood flow; hence alterations in vascular sympathetic innervation are associated with the development and maintenance of cardiovascular diseases [20]. VEGF and its receptors have been reported to promote vascular sympathetic innervation [21,22]. Therefore, VEGF regulates not only the formation of collateral vessels as a potent angiogenic factor in terms of hypoxia or other biochemical and biomechanical forces, but also has a strong influence on the physiology of cardiovascular regulating systems.

4.4. The cardiovascular system and the role of NGF

NGF plays a pivotal role in different scenarios hence on its multifunctional properties. It is known to be the main neurotrophic factor regulating survival and differentiation of SN [18]. Current studies identified NGF also as a pro-survival factor for cardiomyocytes [19]. In addition, NGF promotes angiogenesis in ischemic hind limbs [10] and improves cardiac function after MI [11]. These observations are likely due to a crosstalk regulation between NGF and VEGF. During MI intracardiac cells are exposed to increasing stretch. On the one hand, stretch contributes to NGF depletion in cardiomyocytes [13], whereas SN respond to stretch with an upregulation of various neurotrophins including NGF and CNTF [12]. Whether stretch-induced neuronal VEGF expression plays a beneficial role in different in vivo scenarios like MI remains to be clarified.

Taken together, current studies indicate that VEGF has direct effects on the nervous system in terms of neuronal growth, survival and neuroprotection. Conversely, NGF, a neurotrophin that plays a crucial role in promoting neurotrophic effects in sympathetic neurons, has been identified as a novel angiogenic molecule exerting a variety of effects on endothelial cells and in the cardiovascular system in general. This crosstalk of neurotrophic effects of VEGF and angiogenic effects of NGF has been thoroughly reviewed by Lazarovici et al. [25].

4.5. Neuronal HIF-1α expression is linked to mechanical stretch, whereas KDR expression depends on NGF signaling

We here provide first evidence that stretch of SN leads to HIF-1α upregulation. HIF-1α has been shown to be involved in neuronal VEGF induction, activated by NGF and TrkA signaling [9]. Furthermore, we demonstrated that elongation of SN has no effect on KDR expression, whereas NGF neutralization resulted in a significant increase in neuronal KDR expression. These results may help to understand angiogenic signaling routes in neuronal cells and to identify potential targets to influence angiogenic responses in terms of physiological or pathological circumstances.

4.6. Study limitations

In the present study we used an in vitro model of cultured SN from neonatal rats, which were exposed to a gradual increase of baseline stretch over a time period of 72 h. The expression pattern of VEGF may vary significantly between the cells used in this study and in analogous human cells. Therefore, this study does not claim to resemble the full in vivo scenario as it occurs in the developing and diseased heart. The goal of our study was to characterize the influence of stretch on neuronal VEGF expression on a cellular basis. NGF has also been shown to exert cellular effects via the activation of its low-affinity p75 receptor. In the present study, we did not investigate the role of the p75 receptor.

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