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Sub threshold high frequency electrical field stimulation effect for angiogenesis and vegf expression in cardiomyocytes and heart tissue. Remodelling in the heart: impact of mechanical stretch, threshold and sub-threshold electrical stimulation on cultivated cardiomyocytes and sympathetic neurons

DOCTORAL DISSERTATION

Biomedical sciences,
Medicine (06 B)

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Ikislenkstinio didelio dažnio elektrinio lauko stimuliacijos efektas angiogenezei ir kraujagyslių endotelio augimo veiksnio sintezei kardiomiocituose. Širdies remodeliacija: mechaninio tempimo, slenkstinės ir ikislenkstinės elektrinės stimuliacijos įtaka kardiomiocitams ir simpatinės sistemos neuronams

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CONTENT

1. ABBREVIATIONS

AF - atrial fibrillation
CAD - coronary artery disease
DCM - dilated cardiomyopathy
HCAECs - human coronary artery endothelial cell
HFES - high- frequency bipolar electrical field stimulation
IA -immunoadsorption
IHD - ischemic heart disease
NGF - nerve growth factor
NRVMs - neonatal rat ventricular myocytes
PCI – percutaneous coronary intervention
SES - subthreshold electrical stimulation
SCG - superior cervical ganglia
SN - sympathetic neurons
VEGF - vascular endothelial growth factor
WB - Western blot

2 INTRODUCTION

2.1 Scientific relevance

Arterial occlusive diseases cause serious ischemic diseases in various organs, such as the heart, brain, and leg, and therapeutic angiogenesis is thought to be beneficial for such conditions. Local administration of recombinant angiogenic growth factors, such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF), salvaged ischemic areas of myocardium and hindlimb in animal models. However, the clinical application requires large amounts of these recombinant proteins and is not feasible at this time. Instead of recombinant proteins, use of gene therapy, in vivo transfection of angiogenic growth factor gene, has been attempted to treat these diseases.

VEGF, a dimeric endothelial cell – specific growth factor, is thought to be a principal angiogenic factor that stimulates migration, proliferation, and expression of various genes in endothelial cells. VEGF is synthesized by cells around vasculature and affects endothelial cells as a paracrine factor. The expression of VEGF is upregulated by hypoxia and various cytokines. One part of our present study revealed that low-voltage electrical stimulation of heart cells induced de novo synthesis of VEGF protein and increase proliferation.

The cardiovascular autonomous neural system represents an integrative fiber network that regulates cardiovascular hemodynamic and electrophysiological processes. During normal physiology, the autonomic nervous system represents a short latency responder that quickly compensates for temporary changes of cardiac function (eg, pumping force, blood pressure, and heart rate). However, when compensation becomes permanent during cardiovascular disease (atrial fibrillation, dilatative or ischemic cardiomyopathy and others) specifically sympathetic activation catalyzes a

further deterioration of the disease process. Besides functional effects on heart rate, inotropy, vasomotion, and cardiac electrophysiology, the sympathetic/parasympathetic nervous systems could be markedly affected be the electrical stimulation and produce vide spectrum of biologically active molecules.

2.2. Study hypothesis

Subthreshold electrical stimulation has been shown to induce an improvement of angiogenesis mediated by increased VEGF and NGF protein expression.

2.3 The aim of the study

To evaluate growth factors response to various stimulation techniques in the field of neurocardiology.

2.4. Study tasks

To investigate the effect of sub threshold electrical stimulation on vascular endothelial growth factor regulation in cultured neonatal rat ventricular myocytes, in the aim to reveal new techniques for therapeutic angiogenesis in ischemic heart disease.

To assess the role of autoantibodies in dilated cardiomyopathy inducing the expression of vascular endothelial growth factor in cardiomyocytes. Effect on contractility, vascular endothelial growth factor secretion as a cell response to tension.

To investigate high frequencies and irregularity effects of stimulation on ventricular myocytes and nerve growth factor.

To evaluate the response of sympathetic neurons to mechanical stretching in the evaluation of biologically active molecules: vascular endothelial growth and neuronal growth factors.

2.5. What is new

Up to now some studies has been shown, that high frequency threshold and sub threshold electrical stimulation can induce biologically active molecules production in non-cardiac tissue. Data from this work show, that ventricular myocytes can produce biologically active proteins which has positive effect on cells proliferations and viability.

3. LITERATURE REVIEW

Ischemic heart disease (IHD) due to coronary artery disease (CAD) remains the leading cause of death in the Western world (1). Revascularization of existing arteries and the formation of new collateral vessels may be a promising strategic aim in order to improve the function of organs suffering from hypoxia. Vascular endothelial growth factor (VEGF) plays a pivotal role in angiogenesis and neovascularization. There is accumulating evidence that VEGF is expressed in the heart and that its expression is markedly increased in response to hypoxia or cyclic mechanical stretch (2, 3). These (patho) physiological stimuli recent studies have shown that subthreshold electrical stimulation (SES) augments VEGF expression in cultured skeletal muscle cells. Moreover, in vivo SES has been shown to induce vascular angiogenesis in a hindlimb ischemia model in rats mediated by VEGF (4, 5). Since SES might be delivered to hypoxic cardiac tissue in vivo (e.g., with catheters or wires) this proof-of-principle study investigated the effect of SES on VEGF expression in cultured neonatal rat ventricular myocytes (NRVMs).

Circulating autoantibodies that target diverse myocardial structures have been identified as major predisposing factors for dilated cardiomyopathy (DCM) (6). Patients with DCM show elevated serum levels of vascular endothelial growth factor whose source is unknown (7). Even though a large number of studies suggest that VEGF is beneficial in ischemic cardiomyopathies due to its well-investigated effects on angiogenesis and neovascularisation (8-10), to the best knowledge of the authors there is no data available reporting a beneficial role of VEGF in DCM. In contrast, recent data indicate that an un-physiological expression and secretion of VEGF might be harmful via its pleiotropic effects on all cell types involved in cardiac remodelling. Accumulating data suspect VEGF as a cardiac transcriptional regulator, which is crucially involved in physiological and pathological hypertrophy signalling in cardiomyocytes (11). In this context, Zhou et al. could convincingly demonstrate that VEGF signalling plays a pivotal role in phenylephrine induced cardiomyocyte hypertrophy (12). Additionally, Tsoporis and colleagues have recently shown that VEGF, which is released by cardiomyocytes promotes proliferation of cardiac fibroblasts (13). It is still unclear whether these different observations can be predictably translated into cardiac remodelling. Nevertheless, since VEGF levels are increased in DCM patients, the question raises whether VEGF is just compensatory elevated or it is directly involved in cardiac remodelling pathways due to unknown mechanisms (14-16).

The current options to inhibit or to reverse the process of structural remodelling in the failing heart are limited. Drugs targeting the beta1-adrenergic or the angiotensin signalling pathways can improve survival but have a narrow therapeutic range due to side-effects including bradycardia and/or hypotension (17,18). There is accumulating evidence that the therapy of immunoadsorption (IA) leads to a long-

term improvement of cardiac function in patients suffering from DCM (19-22). Whether autoantibodies in DCM influence cardiac VEGF expression, and whether an un-physiological VEGF secretion might promote remodelling mechanisms due to hypertrophy signalling and cardiac fibroblast proliferation is rather unexplored.

Atrial fibrillation (AF) is associated with a 1.5- to 1.9-fold mortality risk after adjustment for pre-existing cardiovascular conditions (23). Many factors may contribute to this worse outcome like arterial embolism or hemodynamic deterioration. Recently, an irregular ventricular response during AF has been shown to increase sympathetic nerve activity in vivo (24, 25). This raises the hypothesis whether the irregular activation of cardiomyocytes may affect neural growth and activity. Nerve growth factor (NGF) plays a key role in the structural and functional integrity of sympathetic neurons and regulates survival, differentiation and neurite outgrowth (26). For example, in NGF knockout mice the volume of the sympathetic ganglia is remarkably reduced (27). In contrast, overexpression of NGF in the heart results in cardiac hyperinnervation and hyperplasia of stellate ganglia neurons (28). Up to now molecular mechanisms, which regulate cardiac NGF expression are not fully understood.

Mechanical stretch has been shown to increase vascular endothelial growth factor expression in cultured myocytes (29). 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 (30-32). In the heart, mechanical overload contributes to VEGF augmentation via a TGF-beta signalling pathway (33). The heart is comprehensively innervated by the autonomic nervous system including sympathetic and parasympathetic nerve fibbers 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 (34). 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 (35). For instance, in superior cervical ganglia (SCG) of new-born rats, nerve growth factor (NGF) induces capillary sprouting via the release of VEGF (36). The NGF induced VEGF upregulation in SN is mediated by TrkA receptor activation (37). Taken together, these findings indicate that neuronal cells may regulate their own VEGF requirements depending on the pre-existing biomechanical forces in an autocrine/paracrine fashion by a NGF and TrkA signalling pathway. Likewise, NGF has been shown to promote angiogenesis in ischemic hind limbs (38) and to recover cardiac function after myocardial infarction (MI) (39). During MI intracardiac cells are exposed to increasing stretch. Stretch is one important contributor to neuronal NGF expression (40), whereas cardiomyocytes respond to stretch with a down-regulation of NGF

(41). Up to now, it is quite uncertain if the beneficial effects observed by NGF signalling in terms of increasing wall stress (e.g. MI) are due to a direct NGF function or whether stretch-induced NGF secretion promotes neuronal VEGF expression in an autocrine/paracrine fashion subsequently contributing to neovascularisation and recovering heart function.

4. METHODS

4.1. Cell Cultures of Neonatal Rat Ventricular Myocytes (NRVMs)

All animal experiments were approved by the local and state Ethics in Animal Research Committee (University Hospital RWTH Aachen, Germany, TV-No. 10596 A). NRVMs were isolated and cultured as described previously by Simpson et al. with little modifications according to the protocol by Zobel et al. (42-44). After cervical dislocation, hearts were obtained from 1- to 3-day old Sprague – Dawley rats with no consideration to the gender (Charles River, Sulzfeld, Germany) and digested with collagenase II (1.0 mg/ml, 280 U/mg, C2-22; Biochrom, Berlin, Germany), trypsin (0.5 mg/ml, trypsin 1:250, L-11-002; PPA, Colbe, Germany), and 1% penicillin–streptomycin (P-4458; Sigma-Aldrich, Steinheim, Germany). Myocytes were purified from fibroblasts by passage through a Percoll gradient (P-1644; Sigma-Aldrich). Myocytes were plated onto six-well dishes (140685; Nunc, Schwerte, Germany) at a density of 3×10^5 cells per well. Cells were grown in DMEM/Ham's F-12 + l-glutamine (FG-4815; Biochrom) supplemented with 10% horse serum (B15-021; PPA) and 5% fetal bovine serum (A-15-101; PPA). After 24 h, the serum medium was removed, and the cells were washed and maintained in serum-free DMEM/Ham's F-12 + l-glutamine.

Human coronary artery endothelial cells (HCAECs) were grown in endothelial cell growth medium (C-22020; PromoCell GmbH, Heidelberg, Germany) until starting the experiments with NRVM cell-conditioned medium. The tissue used by PromoCell for the isolation of human cell cultures is derived from donors who have signed an informed consent form (this being done by the donor himself, an authorized agent, or a legal agent), which outlines in detail the purpose of the donation and the procedure for processing the tissue.

4.2. Application of Subthreshold Electrical Simulation (SES)

Three days after cell isolation, serum-containing medium was changed to serum-free medium, and thereafter NRVMs were stimulated for 48 h with low-voltage, high-frequency bipolar electrical field stimulation (HFES) using the C-PaceEP external pacing system (IonOptix, Wageningen, The Netherlands). The stimulus strength (0.5 V/cm, impulse duration 1 ms) was chosen to yield an electrical field that was

well below the threshold that elicited contraction of the cultured myocytes. The lowest capture threshold in the different preparations was 1.0 V/ cm. Lack of contraction of the myocytes was checked every 12 h during the experiment by microscopic inspection of the cultures. The frequencies chosen were 5 Hz, 25 Hz, 50 Hz, and 99 Hz. Cell cultures without HFES served as controls.

4.3. ELISA

For VEGF ELISA, each well was normalized to 300,000 cells, and SES steps from 5 Hz up to 25, 50, and 99 Hz were analyzed. The conditioned media was collected at 0, 24, and 48 h after exposure to SES, and protein in the cell culture supernatant was concentrated by using the vivaspin columns from Sartorius (Göttingen, Germany). Samples were assayed by a VEGF ELISA kit from R&D Systems (Minneapolis, MN, USA) with normalized protein amounts according to the manufacturer's instructions. Protein amount was quantified by using the NanoDrop 1000 from Thermo Scientific (Wilmington, DE, USA).

4.4. Western Blot

Western blots (WB) to assess VEGF and KDR proteins were performed with normalized protein amounts. For WB, cells were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0; #9090.2; Carl-Roth, Karlsruhe, Germany), 1% nonident P-40 (#74.385; Merck, Darmstadt, Germany), and 10% glycerol (#50405-1; Biomol, Hamburg, Germany). Cell lysates were cleared by centrifugation at 17,000×*g* for 20 min. Extracts (100 µg) were subjected to electrophoretic separation through a 10% SDS-polyacrylamide gel (SDS #51430-2, Biomol/ polyacrylamide gel #3029.1; Carl-Roth) and subsequently transferred to a polyvinylidene difluoride membrane (#RPN303F; GE-Healthcare Life Sciences, Freiburg, Germany). The membrane was blocked overnight with 5% bovine serum albumin (#01400-1; Biomol) in phosphate buffered saline (#L-1825; Biochrom) containing 0.1% Tween 20 (PBST; #TW0020; Rockland, ME, USA). Primary rabbit anti-GAPDH (1:1,000; #2118; Cell Signaling, Danvers, MA, USA), rabbit anti-VEGF (1:150; 19003-1-AP; Acris Antibodies, Herford, Germany), and rabbit anti-KDR (200 µg/ml, 1:100; sc-315; Santa Cruz, Heidelberg, Germany) were incubated overnight. Blots were washed three times with PBST and incubated with goat anti-rabbit horseradish peroxidase-conjugated antibody (GE Healthcare) for 1 h. Finally, the ECL Advance Western Blotting Detection Kit (GE Healthcare Life Sciences) was used to visualize the bands with the advanced luminescent image analyzer LAS-3000 from Fujifilm (Tokyo, Japan). Relative densitometry analyses of the bands were performed with the Multi-Gauche V3.0 software from Fujifilm.

4.5. Antibodies

Cells were fixed in 4% paraformaldehyde (8.187.15; KMF, Lohmar, Germany), permeabilized with Triton X-100 (50800-1; Biomol), and blocked with 1% BSA (01400-1; Biomol). Cells were incubated with the primary antibody, (1:150) rabbit anti-VEGF (200 µg/ml, 1:150; sc-507; Santa Cruz) mouse anti-Trop T-C (200 µg/ml; sc-20025; Santa Cruz) overnight at 4°C and then with a goat anti-rabbit (1:100) and goat anti-mouse (1:100) secondary antibody for 2 h coupled to Alexa Fluor 488 (VEGF) (1 mg/ml; A11008; Invitrogen) or Alexa Fluor 647 (Trop T-C) (1 mg/ml; A20990; Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochlo- ride (10236276001; Roche Mannheim, Germany). To visualize fluorescence signals, the Axiovert 200M micro- scope and the AxioVision Rel. 4.5 software from Zeiss (Jena, Germany) were used.

Primary antibodies for Western blot: rabbit anti-GAPDH (1:1,000; #2118; Cell Signaling), rabbit anti-VEGF (1:150; 19003-1-AP; Acris Antibodies), rabbit anti-KDR (200 µg/ml, 1:100; sc-315; Santa Cruz).

4.6. RNA Preparation, First-Strand cDNA Synthesis, and Quantitative Real-Time Reverse Transcription-PCR

Total RNA was extracted from NRVMs using the Qiagen RNeasy Mini Kit (Venio, The Netherlands) and following the manufacturer's instructions. A total of 1 µg of RNA were reverse-transcribed using random hexamers from the Fermentas First strand cDNA Synthesis Kit (#K1622; Fermentas GmbH, St. Leon- Rot, Germany).

4.7. Quantitative Real-Time Reverse-Transcription-PCR. Primers and Probes for Quantitative Real-Time Reverse-Transcription-PCR

Real-time PCR was performed in 96-well plates on the Step-One plus Sequence Detection System (ABI) as described previously (9). Data were collected with instrument spectral compensation by Applied Biosystems SDS 1.2.3 software (Applied Biosystems, Foster City, CA, USA).

PCR primers and fluorogenic probes for the target gene and the endogenous control were purchased as Assays-On- Demand (Applied Biosystems). The assay numbers for the endogenous control and target genes were as follows: Rn00560865_m1 (b-2 microglobulin), Rn01533872_m1 (Ngfb), Rn00561661_m1 (Nppa). The assay numbers were as follows: Rn01511602_m1 (VEGF), Rn00564986_m1 (KDR), Rn00560865_m1 (b-2 microglobulin).

4.8. Investigation of Human Coronary Artery Endothelial Cell Proliferation

To reveal the proliferation of HCAECs due to an effect of biologically active VEGF secreted by NRVMs exposed to SES, we used a colorimetric Assay kit from PromoCell (WST-8). WST-8 is bio reduced by cellular dehydrogenases to an orange formazan product that is soluble in cell culture medium. The amount of formazan is directly proportional to the number of living cells. Therefore, this method allows sensitive determination of the number of viable cells in cell culture.

HCAECs were incubated for 48 h with NRVM- conditioned medium. The frequencies of 25 Hz and 5 Hz were analysed, due to the fact that the dominant effect of VEGF induction was observed at 25 Hz (compared to 5 Hz and control).

4.9 DCM patient selection, mechanical stretch and Tunel assay investigation

DCM patients (n 1⁄4 5, mean age 50.3 ± 3.5 years) with severe left ventricular dysfunction (LVEF < 30%, New York Heart Association class III) were treated with IA on five consecutive days. After the final IA session, all patients received 0.5 g/kg polyclonal IgG (Venimmun N) to restore IgG plasma levels (45). The same polyclonal IgG pool was used for cell culture control purposes in a matched concentration, and is indicated as healthy control IgG (HC-IgG) in the appropriate graphs. All DCM patients had a history of heart failure of at least 1 year and had a stable dosage of oral medications including angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, diuretics, aldosterone antagonists and b-blockers for at least 6 months. Ischemic disease was excluded by angiography. The IA therapy was performed with protein A Immunoadsorba columns (Fresenius Medical Care AG, Bad Hombug, Germany). Column eluent (CE) from IA columns containing the eliminated IgG was collected and prepared for cell culture experiments as described elsewhere (22). The Nanodrop ND-1000 (PeqLab, Erlangen, Germany) was used to determine the protein concentration of the CE. Aliquots of CE were stored at 80 C until use. Serum IgG levels of our DCM patients were monitored and an average reduction in IgG levels of 91.7% could be observed, as measured at the end of the last session on the fifth day of IA. Ischemic cardiomyopathy (ICM) patients (n 1⁄4 7) showed similar parameters regarding ejection fraction, LV-dimensions and the NYHA classifications as compared to DCM patients (data not shown).

For mechanical stretch NRCM were incubated on collagen-coated silicone membranes and static stretch was introduced to attached myocytes by applying a gradual increase of tension as described previously (46). Stretch was increased stepwise every 24 h from 3% to 13% (steps: 3, 7, 13%).

The TUNEL assay was performed using the APO-BrdU TUNEL Assay Kit (Invitrogen), according to the manufacturer's instructions. Cell apoptosis was determined

by counting the number of TUNEL- positive nuclei and expressed as percentage of total cells counted (47).

4.10. Statistical Analysis

All values are expressed as mean±SEM. Data were processed using Statistical Package for Social Sciences release 14.0 for Windows (IBM, Somers, NY, USA). The statistical significance of differences was evaluated by the Student's *t*-test for two groups and one-way ANOVA followed by LSD post hoc test for multiple groups. A value of $p < 0.05$ was accepted as statistically significant.

5. RESULTS

5.1. SES Augments VEGF Expression and Secretion in Neonatal Rat Ventricular Myocytes

To analyze the effect of SES on cardiomyocyte VEGF expression, NRVMs in cell culture were stimulated for 48 h at 5 Hz, 25 Hz, 50 Hz, and 99 Hz. VEGF protein amount was measured from cell culture medium at 24 and 48 h of stimulation. HFES elicited an increase in VEGF expression both in the cytoplasm and supernatant. A dominant effect of SES was observed at 25 Hz with a 1.8-fold increase of VEGF protein amount in the cytoplasm (Fig. 1). In parallel, VEGF in the cell culture supernatant rose significantly with an almost bell-shaped dose–response curve and a maximum at 25 Hz. The effect of HFES on VEGF expression was sustained for at least 48 h at all HFES frequencies (Fig. 2). Figure 3 shows VEGF immunocytofluorescence images of the VEGF expression in cardiomyocytes, which were exposed to subthreshold high-frequency electrical fields in vitro.

In order to evaluate which was the relative contribution of augmented VEGF gene expression on elevated VEGF protein expression, we analysed the VEGF mRNA content of cell lysates.

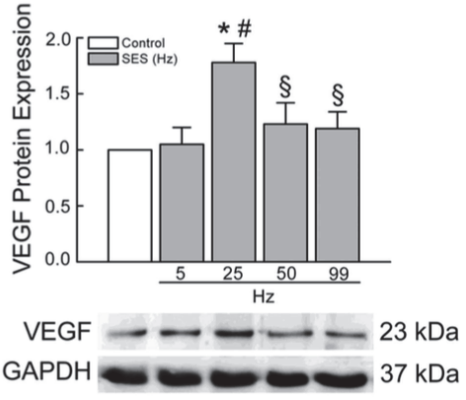

Figure 1. Frequency dependence of relative VEGF protein expression in cell lysates during 48 h of subthreshold electrical stimulation (SES). Results are representative of at least five independent experiments. *p<0.05 versus control. #p<0.05 versus 5 Hz. §$p < 0.05$ versus 25 Hz.

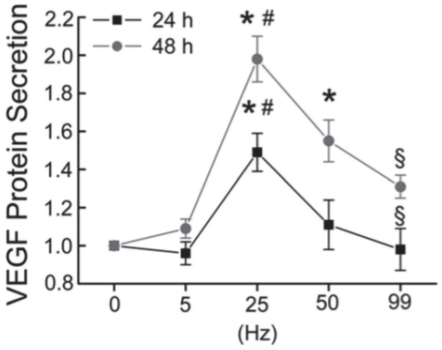

Figure 2. Extracellular secretion of VEGF protein normalized to control. Medium was collected at 24 and 48 h during continuous electrical stimulation, and VEGF protein was determined by ELISA. Results are representative of at least five independent experiments. *p<0.05 versus control. #p<0.05 versus 5 Hz. §p < 0.05 versus 25 Hz.

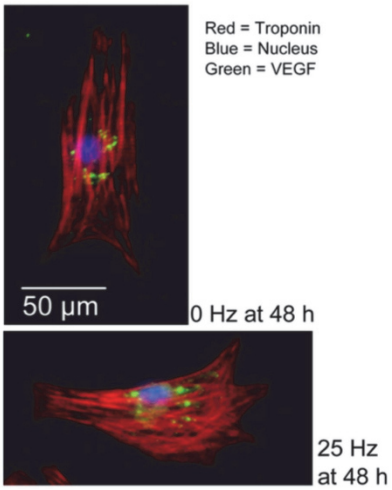

Figure 3. Representative images of NRVMs in cell culture exposed to SES. These images show abundance of VEGF protein.

5.2. Changes of VEGF mRNA and VEGF Receptor During HFES in NRVMs

HFES led to significant decreases in VEGF mRNA, which was significant for 25, 50, and 99 Hz with a maximum at 25 Hz (Fig. 4). Since this might be due to a negative feedback loop in response to the increased VEGF protein levels both in the cytoplasm and in the supernatant, we measured the expression of the membranous VEGF receptor (KDR) both on protein (Fig. 5) and mRNA levels (Fig. 6). SES significantly decreased the expression of the KDR receptor protein at 25 Hz and though not significant at 50 and 99 Hz. Likewise, the KDR receptor mRNA expression was significantly augmented during SES at 25, 50, and 99 Hz (Fig. 6). Of note, the relative suppression of VEGF mRNA expression and KDR protein and gene expression followed the frequency dependency as observed for the induction of VEGF protein with a maximum at 25 Hz.

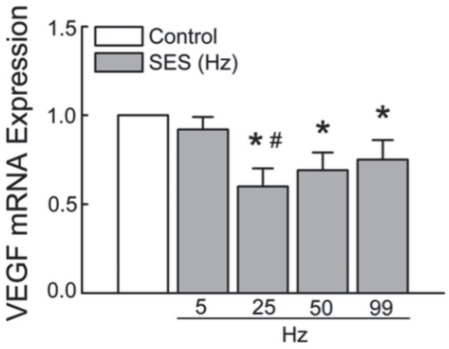

Figure 4. Relative expression of VEGF mRNA in cultured NRVMs. Cells were electrically stimulated for 48 h at indicated frequencies. Thereafter, total cDNA was extracted, and RT-PCR for VEGF mRNA was performed. Results are representative of at least four independent experiments. *p < 0.05 versus control. #p < 0.05 versus 5 Hz.

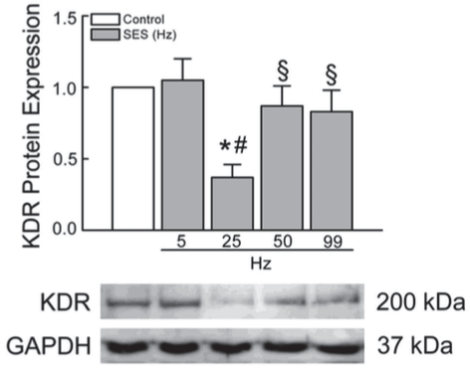

Figure 5. Relative KDR protein expression. Results are representative of at least five independent experiments. *p<0.05 versus control. #p < 0.05 versus 5 Hz. §p < 0.05 versus 25 Hz.

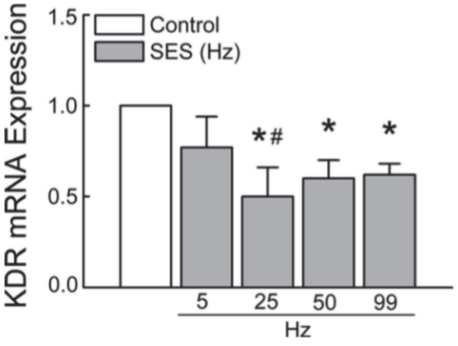

Figure 6. Relative expression of KDR mRNA in cultured NRVMs. Cells were electrically stimulated for 48 h at indicated frequencies. Results are representative of at least four independent experiments. *p<0.05 versus control. #p<0.05 versus 5 Hz.

5.3 SES-Induced VEGF Expression Is Biologically Active and Promotes Human Coronary Artery Endothelial Cell Proliferation

To investigate whether the observed VEGF induction by SES is effective in promoting endothelial cell proliferation, HCAECs were incubated with NRVM-conditioned medium (48 h of SES at 5 Hz, 2 5Hz, 50 Hz, and 99 Hz, respectively). We found that medium conditioned by 25 Hz SES augmented the growth of HCAECs, whereas

the medium conditioned by 5 Hz SES did not elicit a significant growth response of HCAECs in vitro (Fig. 7).

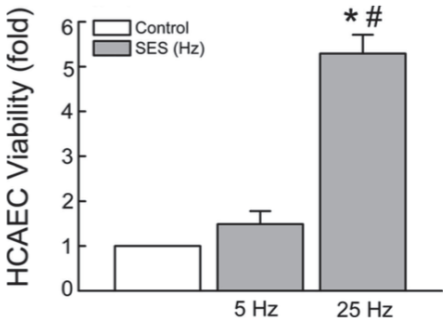

Figure 7. Growth response of HCAECs incubated with cell conditioned medium of NRVMs exposed to SES. Conditioned culture supernatant, 25 Hz, was sufficient to promote endothelial cell proliferation. Results are representative of at least four independent experiments. *p < 0.05 versus control. #p < 0.05 versus 5 Hz.

5.4. Augmented serum levels of VEGF in patients with DCM decrease after IA

Since increased myocardial wall stress can affect VEGF expression, the VEGF serum levels of DCM patients were compared to that of patients with ischemic cardiomyopathy. The left ventricular ejection fraction as well as left ventricular end-systolic and end-diastolic diameters of the two groups showed no significant differences (data not shown). In our small number of patients we observed significantly higher baseline VEGF serum levels in the group of DCM as compared to the patients in the group of ICM. Four weeks after the IA therapy we observed a sizeable decrease of serum VEGF levels in the group of DCM patients compared to the baseline levels (Fig. 8). At this point, the serum VEGF levels in both groups (DCM vs. ICM) showed nearly the same VEGF concentrations, without any significant differences in the above-mentioned echocardiographic parameters.

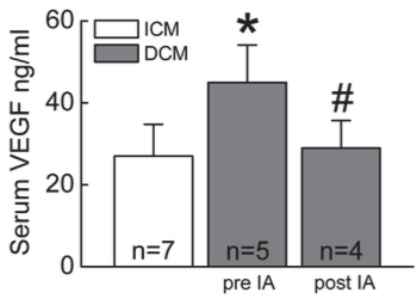

Figure 8. A Serum levels of VEGF decrease after the IA therapy. VEGF serum levels in patients with DCM showed significantly higher baseline values as compared to ICM patients. After the IA therapy, serum VEGF levels showed almost the same concentration in both groups. In our small number of patients, this decrease was not paralleled by statistically significant alterations in echocardiographic parameters (data not shown). The number of patients post IA decreased to n 1⁄4 4, since one of the 5 DCM patients who initially participated the study received a heart transplantation during the study period. *p < 0.05 vs. ICM, #p < 0.05 vs. DCM.

5.5. Pre-treatment of NRCM by DCM-IgG induce the expression of VEGF

In cell culture, we first investigated the dose response of DCM- IgG on NRCM with respect to apoptosis. We observed that the degree of apoptosis increased slightly beyond baseline levels at a DCM-IgG concentration of 500 ng/ml (Fig. 9).

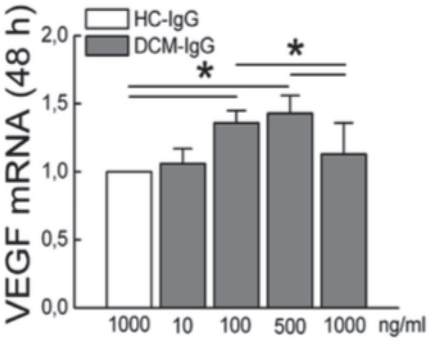

Figure 9. Dose dependent effect of DCM-IgG on the rate of apoptosis in NRCM.NRCM were incubated with healthy control (HC) IgG and DCM-IgG. Apoptosis was determined by TUNEL assay. At a dosage of 500 ng/ml DCM-IgG a slight increase in apoptosis could be observed, which became significant at a concentration of 1000 ng/ml. Pretreatment of NRCM with HC-IgG remained without effect on apoptosis. HC-IgG 1⁄4 polyclonal IgG (Venimmun N) to restore IgG plasma levels, DCM-IgG 1⁄4 IgG from patients with DCM. *p < 0.05 vs. HC- IgG and DCM-IgG 10, 100, 500 ng/ml. All n 1⁄4 5 cell preparations.

Thus, to avoid side effects due to cell death all further experiments were carried out with DCM-IgG concentrations not above a dosage of 100 ng/ml. At this concentration, NRCM did not show an increase in apoptosis, but a significant increase in VEGF expression, both on mRNA and protein levels (Fig. 10 A, B). At a DCM-IgG concentration beyond 500 ng/ml this effect was reversed, with VEGF levels receding to the range of control cells, possibly due to an increase in cell death.

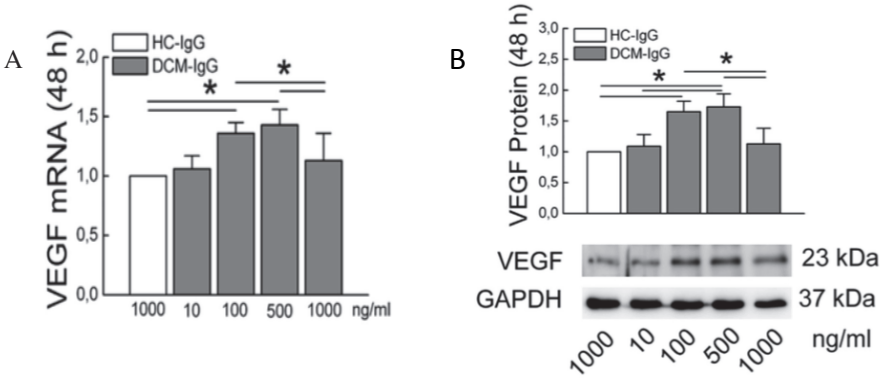

Figure 10 (A, B). The expression of VEGF is increased in NRCM due to DCM-IgG stimulation. Treatment of NRCM with DCM-IgG at a concentration of 100 ng/ml lead to an increase in VEGF expression both on mRNA and protein levels as compared to the group of NRCM treated with healthy control IgG at a concentration of 1000 ng/ml. At a DCM-IgG concentration of 1000 ng/ml VEGF levels decreased probably as a result of cell apoptosis.

Using the ELISA technique the secretion of VEGF in the cell culture supernatant could be investigated in a time dependent manner. At a DCM-IgG concentration of 100 ng/ml, the increase in VEGF secretion became statistically significant after 24 h as compared to 0 h, and after 48 h this difference was significant as compared to the control cells treated by HC-IgG (Fig. 11). NRCM treated with 100 ng/ ml of IgG from healthy donors did not show any relevant alteration of VEGF secretion during a period of 48 h.

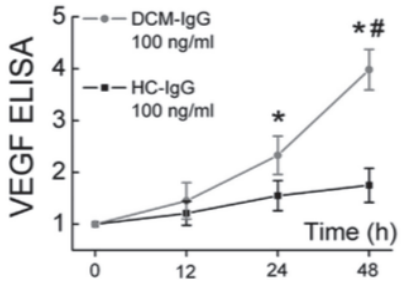

Figure 11. The time dependent increase in VEGF secretion due to DCM-IgG stimulation was determined by ELISA experiments. A significant increase in VEGF secretion could be observed after 24 h of 100 ng/ml DCM-IgG stimulation as compared to 0 h. After 48 h of 100 ng/ml DCM-IgG stimulation the increase in VEGF expression became also significant if compared to the control cell group, which was stimulated by HC-IgG at the same concentration of 100 ng/ml. A, B: *p < 0.05, C: *p < 0.05 vs. 0 h, #p < 0.05 vs. control cells. All n 1⁄4 5 cell preparations.

5.6. Mechanical stretch, but not contraction rate increases VEGF expression in NRCM

DCM is accompanied by an increase in heart rate and mechanical overload. Therefore we analyzed the effects of both parameters on the expression of VEGF on a cellular basis. At a DCM-IgG con- centration of 100 ng/ml a significant increase in contraction rate of NRCM could be observed by microscopic examination (Fig. 12). Higher DCM-IgG concentrations resulted in a decrease in contraction rate, probably due to cell damage ultimately leading to apoptosis, whereas control cells that were incubated with equal IgG concentrations from healthy donors remained unaffected.

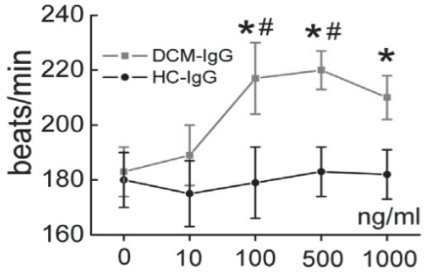

Figure 12. Mechanical stretch, but not contraction rate contributes to the increase in VEGF expression in NRCM. The contraction rate of cultured cardiac myocytes increased dose dependently with a maximum at 100 ng/ml of DCM-IgG stimulation.

Pretreatment of NRCM with metoprolol, a selective beta1-adrenergic receptor blocker, resulted in a decrease in contraction rate without any influence on VEGF expression (Fig. 13 A, B).

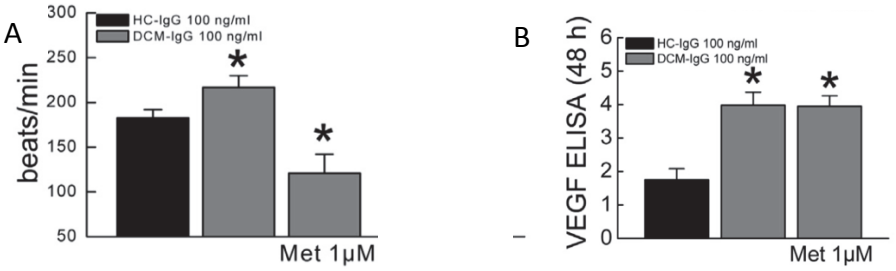

Figure 13. Blockade of the beta-1 adrenergic signalling by metoprolol decreased the contraction rate without any effect on VEGF expression (A, B).

By contrast, mechanical stretch lead to an increase in NRCM VEGF expression (Fig. 14). Coincubation of stretched cells with DCM-IgG at a concentration of 100 ng/ml showed an additive effect on VEGF induction (VEGF protein expression; control: 1 ± 0.0 vs. stretch 13%: 1.75 ± 0.15 ($P < 0.05$) vs. stretch 13% þ 100 ng/ml DCM-IgG: 1.95 ± 0.23).

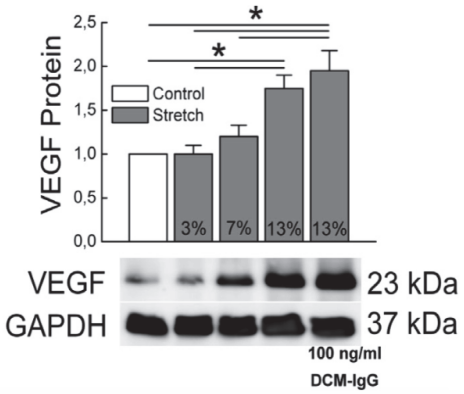

Figure 14. Mechanical stretch-induced VEGF induction was enhanced by DCM-IgG stimulation (D, right panel 13% stretch with 100 ng/ml DCM-IgG). Met 1⁄4 Metoprolol. A: *p < 0.05 vs. 0 ng/ml of DCM-IgG, #p < 0.05 vs. HC-IgG. B, C: *p < 0.05 vs. HC-IgG, #p < 0.05 vs. DCM-IgG. D: *p < 0.05. All n 1⁄4 5 cell preparations.

5.7. Arrhythmic stimulation increases NGF expression in ventricular myocytes

We first analyzed NGF mRNA expression in cardiomyocytes exposed to increasing frequency (0, 5 and 50 Hz) and irregular stimulation (5, 25 and 50% SD). At a constant rate of 5 Hz, we observed a slight but insignificant increase of NGF expression at a low degree of irregularity (5% SD). The increase in NGF mRNA expression became significant as irregularity rose to 25% SD (Fig. 15). During irregular stimulation with 50% SD NGF mRNA expression returned to control levels. By contrast, at

a constant rate of 50 Hz NGF mRNA expression decreased significantly independent of the applied irregularity. Variability up to 50% SD remained without effect during stimulation with 50 Hz (Fig. 16).

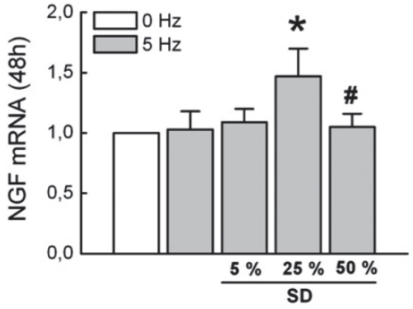

Figure 15. Expression of NGF in ventricular myocytes depends on stimulation rate and irregularity. Panel demonstrate that an irregular stimulation with 25% SD significantly increases NGF mRNA expression in ventricular myocytes.

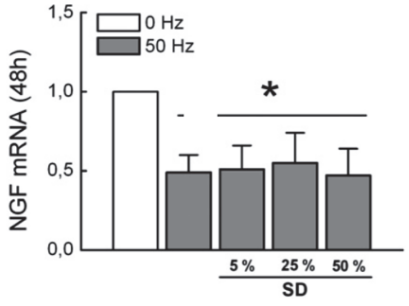

Figure 16. As frequency rises, NGF mRNA expression decreases in a significant manner. All PCR data are derived from at least n=3 experiments. *P<0.05 vs. 0 Hz. #P<0.05 vs. 5 Hz+25% SD or 50 Hz.

Next, we performed highly sensitive ELISA experiments with cardiomyocyte cell-conditioned medium to analyze the secretion of NGF in the cell culture supernatant. We found that the increase in NGF mRNA expression by 5 Hz at 25% SD was paralleled by higher NGF levels in the cell culture supernatant (Fig. 17). On the other hand, HFES with 50 Hz significantly decreased the amount of NGF in the cell culture supernatant (Fig. 18). This effect was not altered by irregularity. The ELISA data could be confirmed by Western blot experiments. Arrhythmic stimulation significantly increased NGF protein levels in cardiomyocytes.

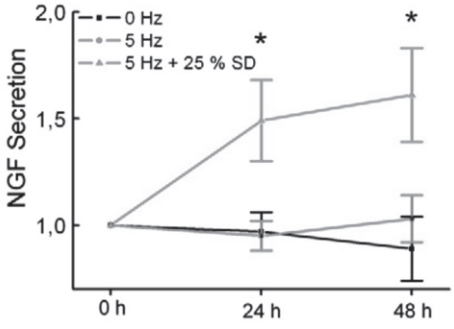

Figure 17. NGF mRNA increase is accompanied by a higher NGF secretion into the cell culture supernatant. ELISA experiments revealed higher NGF levels in the cell culture supernatant as irregularity rose to 25% SD at a constant rate of 5 Hz. These experiments also revealed time dependency. NGF increase showed slightly higher values at 48 h as compared to 24 h.

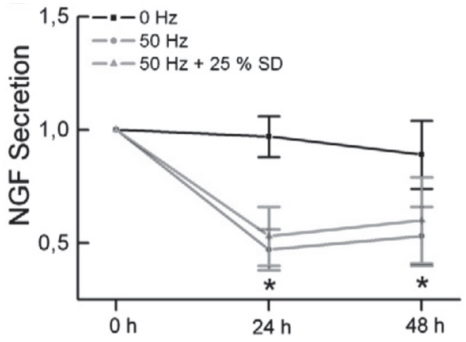

Figure 18. HFES with 50 Hz over a stimulation period of 48 h decreased NGF levels in the cell culture supernatant significantly. This response peaked at 24 h with a slight increase after 48 h of HFES stimulation. Irregularity with 25% SD showed no significant effect on NGF secretion. All ELISA data are derived from at least n = 3 experiments. *P < 0.05 vs. 0 Hz. #P<0.05 vs. 5 Hz+25 SD or 50 Hz+25% SD.

5.8. The NGF increase during arrhythmic stimulation returns to control levels after cessation of the arrhythmia

Fig. 19 depicts cardiomyocytes in cell culture, double stained for troponin-t (red) and NGF (green). This image demonstrates the abundance of NGF that is expressed by ventricular myocytes. To analyze, whether the effect of arrhythmic pacing on NGF expression in cardiomyocytes is sustained after cessation of the irregular stimulation, we performed further experiments using an altered stimulation program. After an initially arrhythmic stimulation with 5 Hz at 25% SD for the first 24 h, we

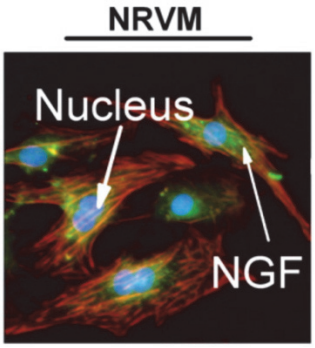

Figure 19. IF images showing NRVM with NGF expression and SCG cell cultures with GAP-43 expression. Panel A demonstrates the abundance of NGF expressed by ventricular myocytes.

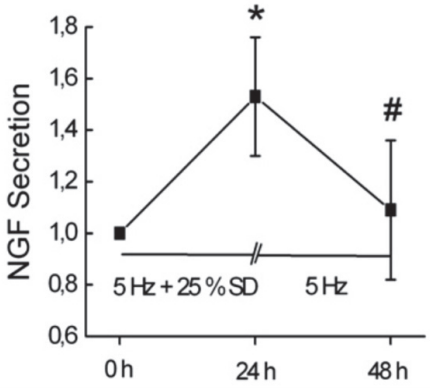

Figure 20. The levels of NGF in the cell culture supernatant of 5 Hz + 25% SD stimulated NRVM cells are sufficient to promote neurite outgrowth of cultured sympathetic neurons (C, D). ELISA and WB data are derived from at least n=3 cell preparations. *P<0.05 vs. 5 Hz+25% SD at 0 h (ELISA) or 5 Hz at 48 h (WB). #P<0.05 vs. 5 Hz+25 SD at 24 h (ELISA).

switched off the irregular stimulation and continued at 5 Hz without the variability of 25% SD for further 24 h. We found that the initial (first 24 h) increase in NGF expression under conditions of arrhythmic pacing returned almost to baseline levels after termination of the irregular stimulation (Fig. 20).

5.9. Increased NGF levels due to arrhythmic stimulation are sufficient to promote neurite outgrowth in sympathetic neurons

To determine whether the increase in cardiomyocyte NGF expression and secretion in terms of irregular stimulation is sufficient to in- duce neurite outgrowth in sympathetic neurons, primary cultures of sympathetic neurons were incubated for 48 h with cardiomyocyte cell-conditioned medium that were collected after 48 h of electrical stimulation. The higher amount in NGF protein levels, resulting from arrhythmic stimulation contributed to an increase in neurite outgrowth in a significant manner as shown by Immunocytofluorescence and Western blot experiments for GAP-43 protein expression, an established marker for neuronal growth (Fig. 21A, B).

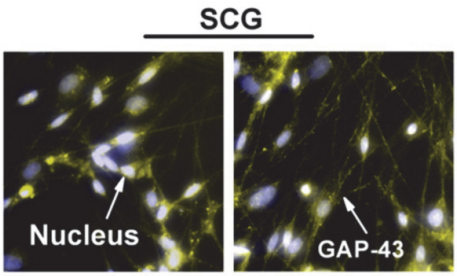

Figure 21 A. The levels of NGF in the cell culture supernatant of 5 Hz + 25% SD stimulated NRVM cells are sufficient to promote neurite outgrowth of cultured sympathetic neurons.

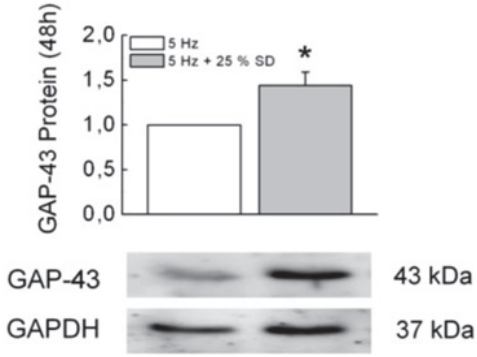

Figure 21B. WB data are derived from at least n=3 cell preparations. *P<0.05 vs. 5 Hz+25% SD at 0 h or 5 Hz at 48 h (WB).

5.10. 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. 22).

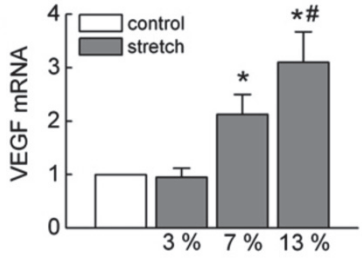

Figure 22. Mechanical stretch of SN increases VEGF mRNA and protein expression in a time and dose depended manner. SN were exposed to a gradual increase of mechanical stretch over a time period of 72 h. (A) Real-time PCR showed a time and dose depended increase in VEGF mRNA expression by stretch up to 13%.

The higher VEGF mRNA content was paralleled by an increase in VEGF protein expression as confirmed by Western blotting (Fig. 23).

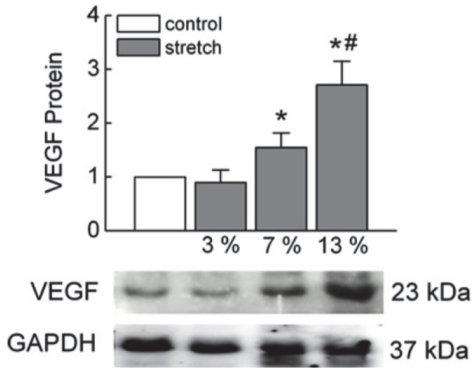

Figure 23. The increase in mRNA expression was paralleled by an almost 2,5-fold increase in VEGF protein expression after 72 h of stretch by 13%.

Morphological nerve sprouting due to stretch by 13% could be detected by microscopical images as shown in Fig. 24.

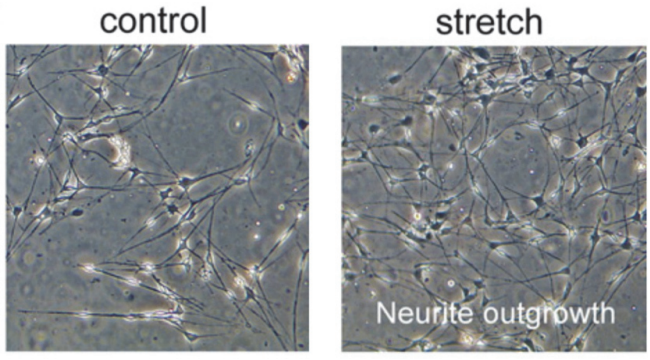

Figure 24. Microscopic images showing control and stretched neurons in cell culture.

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. From 24 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% (data not shown).

5.11. 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. 25).

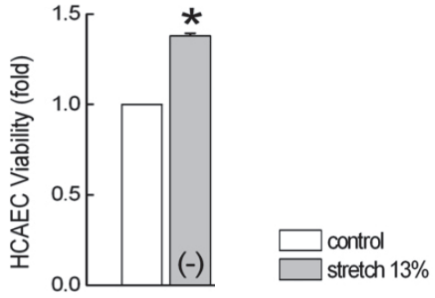

Figure 25. To demonstrate the biological activity of neuronal VEGF secreted in terms of mechanical stretch, endothelial cells from human coronary arteries (HCAEC) were incubated with SN cell-conditioned medium for 48 h.

6. DISCUSSION

The principal findings of our study are (a) SES of cultured NRVMs elicits a significant increase in VEGF protein expression, (b) the dominant frequency of VEGF induction by SES was 25 Hz, (c) NRVM VEGF secretion due to SES is biologically active and promotes HCAEC proliferation in vitro.

The most dominant effect of SES on VEGF expression was present at 25 Hz with an almost twofold increase in extracellular VEGF protein levels. A frequency dependency of VEGF expression during in vitro SES was previously described in other cell lines with the optimal electrical frequency varying for different cell types, that is, 50 Hz for skeletal muscle cells and 24 Hz for smooth muscle cells (48). The exact mechanism by which HFES augments cellular protein expression is unclear. In myocardial or cardiac neuronal cells, the functional excitatory effects of electrical stimulation (e.g., muscle cell contraction or neuronal firing) depends on the membrane depolarization, which in turn is determined by its refractory period, with higher frequencies being typically effective in cardiac neuronal cells due to

its shorter refractory periods. Trophic effects of SES have also been described in sympathetic or parasympathetic cardiac neuronal cells (reference our group). High-frequency stimulation led to an increased NGF and NT-3 protein and gene expression with subsequent induction of neuronal cell growth and hypertrophy in vitro and in vivo (49). However, as opposed to the present study, this effect was dependent on neuronal cell membrane depolarization, since suprathreshold current was delivered, and the effect was blunted by lidocaine (50).

In this study, subthreshold electrical stimuli that were not capable of myocardial depolarization. Thus, different electrical effects than only cell membrane depolarization may be operative.

Most notably, SES seems to represent a ubiquitous mechanism by which cellular expression of growth factors can be enhanced. This is further supported by the observation of a frequency-dependent increase in insulin- like growth factor-2 protein and gene expression in osteosarcoma cells (51). In fact, this effect may not solely be restricted to the induction of growth factors, since an increase in constitutive NO synthase during SES of cardiomyocytes has also been described (48).

Theoretically, SES could increase the stability of VEGF protein. However, Kanno et al. showed that electrical stimulation did not affect the stability of VEGF mRNA. This is evidence that the augmentation of VEGF mRNA by electrical stimulation occurs predominantly at the transcriptional level. In addition, transient electrical stimulation (2 h vs. 24 h) led to almost identical VEGF mRNA levels indicating that the initial mRNA increase elicited by SES was sufficient to induce a sustained augmentation of VEGF protein synthesis. This is further sup- ported by the observation of a later onset of the increase in VEGF protein expression 12 h after initiation of SES with a subsequent maximum at 48 h (4).

In the present study, the VEGF mRNA amount was decreased after 48 h of SES despite an increase in VEGF protein. Since KDR receptor mRNA and protein were decreased, a negative feedback mechanism of increased VEGF protein levels on VEGF mRNA expression via downregulated KDR expression might be operative. In fact, Hang and colleagues have already shown that the VEGF mRNA expression gradually decreases during prolonged electrical stimulation and that VEGF-induced angiogenesis may be under a negative feedback control (52). Thus, if a limited exposure of SES is capable of inducing an increase in VEGF mRNA sufficient to induce a sustained VEGF protein expression, the refectory downregulation of VEGF mRNA expression may already occur within the 48-h stimulation period, thus yielding overall lower mRNA levels than in the control group.

Of note, a negative feedback loop secondary to SES - induced VEGF upregulation might counteract the beneficial effect of SES on therapeutic angiogenesis. Since, in a potential clinical application scenario, SES may only be applied on a short-term basis (e.g., via an intracardiac wire or catheter), the induction of angiogenesis may still be sufficient for a clinically meaningful effect on revascularization. Such a hy-

pothesis is supported by the present study in which a short-term SES exposure of 48 h led to a VEGF protein increase sufficient to induce a cellular growth in vitro.

There are several compelling studies showing the beneficial effects of chronically applied electrical field stimulation to various cell culture and animal models. A study by Shafy and colleagues demonstrated that chronic electrical stimulation via a cardiac resynchronization therapy device contributes to cell differentiation of autologous cultured myoblasts, injected into the infracted areas in a sheep model of myocardial infarction. The authors could show that this technical approach resulted in an improvement in myocardial function (53). Recently Haneef et al. furthermore demonstrated that electrostimulation of stem cells is a safe and effective approach for cell survival and differentiation into cardiac cells (54). Regarding the role of VEGF, Spadaccio and coworkers revealed in an in vivo model of myocardial infarction that continued electrical stimulation contributes to an improvement in myocardial function paralleled by an increase in angiogenesis through endothelial progenitor cell migration and VEGF production (43). These novel studies encourage the investigation of myocardial tissue engineering to develop new therapeutic tools for ischemic heart disease. Our results are completely in line with these findings. Moreover, in the field of new therapeutic approaches for the treatment of heart diseases, it is likely safer to have the opportunity to perform SES to avoid side effects like arrhythmias.

Autoimmune mediated angiogenic signaling has been described e.g. in systemic lupus erythematosus or myasthenia gravis (55, 56). In the latter, the authors could demonstrate a relevant positive correlation of VEGF levels in patients with myasthenia gravis as compared to healthy controls. Furthermore, in cancer research pro- and anti- angiogenic factors are known to be crucially involved in tumor progression and disease development (57, 58). VEGF is the main potent angiogenic factor responsible for the formation of new vessels. Up to now, the induction of VEGF in cancer cells is not fully understood. Recently, Lombardi and colleagues could convincingly demonstrate that breast cancer IgG increased the expression of VEGF in tumor cells. This response was promoted via muscarinic receptor signaling. These findings and our present results clearly demonstrate that autoantibodies in various diseases and pathological circumstances are potent inducers of growth factors, especially VEGF (59).

VEGF and its two receptors VEGFR-1 and VEGFR-2 play a pivotal role in angiogenesis under physiological or pathological circumstances and are involved in neuronal and myocardial development and morphogenesis (60). Studies in animal models revealed that even small perturbations in VEGF levels affect cardiovascular development and embryonic survival (61,62). Abnormal VEGF conditions may favor the initiation and progression of cardiac remodeling due to hypertrophy of cardiomyocytes (11,12). Furthermore it appears of great importance that VEGF, which is released by cardiomyocytes enhances cardiac fibroblast proliferation in a paracrine manner (13). A pathological proliferation of fibroblasts may favor the progression of myocardial fibrosis that might further trigger VEGF induction due to reduc-

tion in oxygen tension as a result of microcirculatory disorders. This vicious cycle might perpetuate the progression of pathological remodeling driven by an undirected and non-physiological VEGF release. This consideration is further enhanced by the finding that mechanical overload, an ubiquitous stimulus in DCM, also leads to an increase in VEGF expression by cardiomyocytes that is further enhanced by such autoantibodies.

The present study extends the knowledge about the possible link between autoimmune signaling in DCM and VEGF induction in cardiomyocytes. This novel finding may contribute to new pharmacological approaches to modulate myocardial remodeling in DCM on a molecular basis.

NGF is not only the major neurotrophic factor for the survival and differentiation of sympathetic neurons, it even functions as a pro-survival factor for cardiomyocytes that regulates apoptosis (63) and plays a pivotal role in cardiac repair following myocardial infarction (64). Thus, NGF seems to be a double-edged sword, since pathological NGF regulation is also involved in nerve sprouting and sudden cardiac death (65). The mechanisms by which cardiac NGF expression is up- or downregulated are poorly understood despite its obvious crucial role in the neurophysiology of the heart. Recently, our group could show that mechanical stretch, an ubiquitous stimulus in cardiac tissue contributes to hypertrophy and NGF downregulation in cardiomyocytes via the Cn-NFAT pathway (66).

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 (40, 41). In the present study we showed that stretch of SN goes along with VEGF induction in a time and dose dependent fashion. NGF 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. As one cellular source, the single cardiomyocyte responds to stretch with an upregulation of VEGF (29). 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 (34).

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 (67). VEGF and its receptors have been reported to promote

vascular sympathetic innervation (68, 69). 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.

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 (70). Current studies identified NGF also as a pro-survival factor for cardiomyocytes (71). In addition, NGF promotes angiogenesis in ischemic hind limbs (10) and improves cardiac function after MI (39). 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 (41), whereas SN respond to stretch with an upregulation of various neurotrophins including NGF and CNTF (40). 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. (72).

More details of related scientific observations are provided and summarized at the provided original articles.

7. CONCLUSIONS

- Cardiomyocytes stimulated by low-voltage electrical fields produce potent amounts of the angiogenetic factor VEGF, which has positive proliferation effects on HCAECs in vitro. This is a very potent step forward to the treatment of IHD. Angiogenesis due to SES may be one optional alternative treatment of CAD.
- The IA therapy decreases elevated VEGF serum levels in patients with DCM. DCM-IgG induce VEGF expression in cultured NRCM. Stretch-induced VEGF production is further enhanced by such autoantibodies, whereas the increase in contraction rate due to DCM-IgG induced beta1-adrenergic stimulation does not seem to play a role in VEGF induction.
- Cardiomyocytes control their local NGF under conditions of high rate or irregular stimulation.

- SN respond to stretch with an increase in VEGF expression and secretion. Neuronal VEGF augmentation due to stretch shows a time and dose dependency.

8. STUDY LIMITATIONS

We did not investigate the mechanisms by which SES - induced VEGF augmentation affected the decrease in VEGF mRNA (negative feedback loop). Future experiments using KDR receptor antibodies during SES and analysis of the intracellular signaling cascades leading to the downregulation of VEGF mRNA may further elucidate these mechanisms.

In the present study, we used an in vitro model of cultured CM from neonatal rats. The expression pattern of VEGF may vary significantly between neonatal and adult rat cardiomyocytes. In addition, we did not analyze the SES response in hypoxic myocytes, as they may be found in the infarction border zone.

Therapeutic angiogenesis after myocardial infarction remains a promising, but challenging, task for cardiovascular medicine. Its importance stems from the high morbidity and mortality due to sequels of occlusive CAD like arrhythmias and heart failure. Several attempts to induce angiogenesis have been investigated (like stem cells, local administration of recombinant angiogenetic growth factors: fibroblast growth factor, VEGF).

The current proof-of-concept study shows that SES is capable of boosting endogenous VEGF expression in ventricular myocytes, which in turn is able to induce proliferation of endothelial cells, thus potentially inducing angiogenesis. SES may be delivered via stimulating catheters positioned either at the endocardial site of the LV or via stimulating wires, which may be introduced into the coronary artery (e.g., after revascularization or even in nonoccluded small vessels not amenable to PCI) or accompanying venous vessels (coronary sinus tributaries). The fact that this approach does not rely on the introduction of cells or proteins in myocardial targets by injection or infusion and stimulation from a single electrode pair may cover a larger area makes this approach potentially attractive for further in vivo studies in animal models of CAD.

9. SUMMARY

Santrumpos

BSADEL – bipoliarinė stimuliacija aukšto dažnio elektros lauku
DKMP – dilatacinė kardiomiopatija
IA – imunoadsorpcija
IES – ikislenkstinė elektrinė stimuliacija
IŠL – išeminė širdies liga
KEAV – kraujagyslių endotelio augimo veiksnys
NAV – nervų augimo veiksnys
PKI – perkutaninė koronarinė intervencija
PV – prieširdžių virpėjimas
SSN – simpatinės sistemos neuronai
VAL – vainikinių arterijų liga
VKG – viršutiniai kaklo ganglijai
WB – *Western Blot*
ŽNSM – žiurkių naujagimių skilvelių miocitai
ŽVAEL – žmogaus vainikinių arterijų endotelio ląstelės

1. ĮVADAS

1.1. Tyrimo hipotezė

Ikislenkstinė elektrinė stimuliacija skatina angiogenezės vystymąsi dalyvaujant KEAV ir NAV baltymams.

1.2. Tyrimo tikslas

Įvertinti augimo veiksnių atsaką į įvairias stimuliavimo technikas neurokardiologijos srityje.

1.3. Tyrimo uždaviniai

Ištirti ikislenkstinės elektrinės stimuliacijos poveikį kraujagyslių endotelio augimo veiksnio reguliavimui dirbtinai užaugintuose žiurkių naujagimių skilvelių miocituose, siekiant atskleisti naujas terapinės angiogenezės technikas išeminės širdies ligos atvejais.

Įvertinti autoantikūnų vaidmenį dilatacinės kardiomiopatijos atvejais, indukuojant kraujagyslių endotelio augimo veiksnio raišką kardiomiocituose; įvertinti poveikį kontrakcijai ir kraujagyslių endotelio augimo veiksnio sekrecijai kaip ląstelių atsaką į tempimą.

Ištirti didelių dažnių, nereguliarios stimuliacijos poveikį kraujagyslių miocitams ir nervų augimo veiksniui.

Įvertinti simpatinės sistemos neuronų atsaką į mechaninį tempimą vertinant biologiškai aktyvias medžiagas: kraujagyslių endotelio augimo ir neuronų augimo veiksniai.

1.4. Tyrimo naujumas

Iki šių dienų atlikti tyrimai parodė, kad aukšto dažnio slenkstinė ar ikislenkstinė elektrinė stimuliacija gali sukelti biologiškai aktyvių molekulių gamybą ne širdies audiniuose. Šio tyrimo duomenys rodo, kad skilvelio miocitai gali gaminti bioaktyvius baltymus, turinčius teigiamos įtakos ląstelių proliferacijai ir gyvybingumui.

2. LITERATŪROS APŽVALGA

Vainikinių arterijų ligos (VAL) sukelta išeminė širdies liga (IŠL) išlieka pagrindine mirties priežastimi Vakarų pasaulyje (1). Esamų arterijų revaskuliarizacija ir naujų kolateralinių kraujagyslių susidarymas gali būti perspektyvus strateginis tikslas, siekiant pagerinti organų su hipoksiniais pažeidimais funkciją. Pagrindinį vaidmenį angiogenezės ir naujų kraujagyslių susidarymo procese vaidina kraujagyslių endotelio augimo veiksnys (KEAV). Pateikiama vis daugiau įrodymų, kad KEAV sintezė vyksta širdyje ir jo raiška ženkliai padidėja esant hipoksijai ar ciklišKam mechaniniam tempimui (2, 3). Šių (pato)fiziologinių stimulų tyrimai pastaruoju metu parodė, kad ikislenkstinė elektrinė stimuliacija (IES) padidina KEAV sintezę dirbtinai išaugintose skeleto raumenų ląstelėse. Be to, naudojant žiurkių užpakalinių galūnių išemijos modelį su KEAV, nustatyta, kad IES *in vivo* skatina kraujagyslių angiogenezę (4, 5). Kadangi IES gali būti taikoma hipoksiniam širdies audiniui *in vivo* (pvz., kateteriais ar vielomis), šio kontrolinio-klinikinio tyrimo metu buvo tiriamas IES poveikis KEAV sintezei dirbtinai išaugintuose žiurkių naujagimių skilvelių miocituose (ŽNSM).

Nustatyta, kad cirkuliuojantys autoantikūnai, veikiantys įvairias miokardo struktūras, yra pagrindinis dilatacinės kardiomiopatijos (DKMP) rizikos veiksnys (6). Pacientams su DKMP nustatyta padidėjusi kraujagyslių endotelio augimo veiksnio koncentracija kraujo serume ir tokio padidėjimo šaltinis nėra žinomas (7). Nors, remiantis daugelio tyrimų rezultatais, KEAV yra naudingas išeminės kardiomiopatijos atvejais dėl savo plačiai ištirto poveikio angiogenezei ir neovaskuliarizacijai (8-10), autorių žiniomis, duomenų apie teigiamą KEAV vaidmenį DKMP atvejais nėra. Priešingai, pastarųjų metų duomenys rodo, kad nefiziologinė KEAV ekspresija ir sekrecija gali būti žalinga dėl pleiotropinio poveikio visoms širdies remodeliacijoje dalyvaujančių ląstelių rūšims. Pateikiama vis daugiau duomenų, kad KEAV gali būti širdies transkripcijos reguliatorius, darantis esminį vaidmenį perduodant fiziologinės ir patologinės hipertrofijos signalus kardiomiocituose (11). Šiuo aspektu Zhou ir kt. galėtų įtikinamai pademonstruoti, kad KEAV yra lemiamas veiksnys, rodantis fenilefrino sukeltą kardiomiocitų hipertrofiją. (12). Tsoporis su kolegomis neseniai atlikti tyrimai taip pat atskleidė, kad kardiomiocitų sintezuojamas KEAV skatina širdies fibroblastų plitimą (13). Vis tik nėra aišku, ar šiuos skirtingus pastebėjimus

galima prognostiškai panaudoti širdies remodeliacijai. Tačiau, kadangi pacientų su DKMP KEAV lygis yra padidėjęs, kyla klausimas, ar KEAV padidėjimas yra tik kompensacinis, ar tai yra nežinomu mechanizmu tiesiogiai susiję su širdies remodeliacija (14-16).

Dabartinės galimybės sustabdyti ar pakeisti širdies struktūros remodeliaciją esant širdies nepakankamumui yra ribotos. Nors beta1-adrenerginiai ar angiotenzino blokatoriai gali pagerinti išgyvenamumą, bet jų terapinis taikomumas yra gana siauras dėl sukeliamo pašalinio poveikio, įskaitant bradikardiją ir (arba) hipotenziją (17,18). Pateikiama vis daugiau įrodymų, kad gydymas imunoadsorpcijos (IA) metodu ilgam pagerina širdies funkciją pacientams, kuriems nustatyta DKMP (19-22). Tačiau yra mažai tyrinėta, ar autoantikūnai DKMP atveju turi įtakos širdies KEAV ekspresijai ir ar nefiziologinė KEAV sekrecija gali skatinti remodeliacijos mechanizmus dėl hipertrofijos signalų ir širdies fibroblastų proliferacijos.

Teigiama, kad prieširdžių virpėjimas (PV) padidina mirtingumo riziką nuo 1,5 iki 1,9 karto (23). Prastesnes išeitis, tokias kaip arterijų embolija arba hemodinamikos sutrikimai, gali sąlygoti daugelis veiksnių. Pastaraisiais metais nustatyta, kad nereguliarus skilvelių atsakas PV metu padidina simpatinės nervų sistemos aktyvumą *in vivo* (24, 25). Todėl keltina hipotezė, kad nereguliari kardiomiocitų aktyvacija gali turėti neigiamos įtakos nervų augimui ir nervų sistemos aktyvumui. Struktūriniam ir funkciniam simpatinės sistemos neuronų vientisumui labai svarbus nervų augimo veiksnys (NAV), reguliuojantis išgyvenamumą, diferenciaciją ir aksonų ataugimą (26). Pavyzdžiui, pelių su „išjungtu“ NAV simpatinės sistemos ganglijų kiekis ženkliai sumažėja (27). Ir priešingai, per didelė KEAV ekspresija širdyje sukelia širdies inervacijos padidėjimą ir žvaigždinių mazgų (ganglijų) neuronų hiperplaziją (28). Molekuliniai mechanizmai, reguliuojantys širdies NAV sintezę, ir šiandien nėra visiškai aiškūs.

Nustatyta, kad mechaninis tempimas padidina kraujagyslių endotelio augimo veiksnio koncentraciją dirbinai išaugintuose miocituose (29). Apart biomechaninių jėgų, atsirandančių besivystančioje ar nesveikoje širdyje, audinių, tokių kaip plaučiai, skeleto raumenys, ląstelės ir netgi su neoplastine geneze susijusios ląstelės patenka į biomechaninę aplinką, kurioje vyksta normos ribas viršijantys tempimai (30-32). Mechaninė širdies perkrova prisideda prie KEAV padidėjimo per transformuojančio augimo veiksnio beta (TAF-β) signalų perdavimo kelius (33). Širdį visapusiškai inervuoja autonominė nervų sistema, įskaitant simpatinės ir parasimpatinės nervų sistemos nervines skaidulas, kontroliuojančias įvairius širdies reguliavimo mechanizmus. Taigi, atradimas, kad šie simpatinės sistemos neuronai (SSN) gali gaminti ir išskirti angiogeninius veiksnius, tarp jų ir KEAV, yra neabejotinai reikšmingas (34). Tačiau vis dar nėra išaiškinta, ar tempimas taip pat padidina ir neuroninę KEAV raišką.

Be daugialypio poveikio endotelio ląstelėms, dėl kurių susiformuoja naujos kolateralinės kraujagyslės, KEAV tiesioginis poveikis SSN taip pat yra daugialypis (35). Pavyzdžiui, nustatyta, kad nervų augimo veiksnys, išskirdamas KEAV, sukelia

žiurkių naujagimių viršutinių kaklo ganglijų kapiliarų susidarymą (36). NAV sukeltas KEAV aktyvumo padidėjimas SSN yra susijęs su TrkA receptoriaus aktyvavimu (37). Apibendrinant, minėti rezultatai rodo, kad neuronų ląstelės gali reguliuoti savo pačių KEAV poreikį NAV ir TrkA signalų perdavimo keliais, priklausomai nuo ankstesnių autokrininiu ar parakrininiu būdu veikusių biomechaninių jėgų. Be to, nustatyta, kad NAV skatina angiogenezę (38) ir širdies funkcijos atsistatymą po miokardo infarkto (MI) (39). MI metu intrakardinės ląstelės vis labiau tempiamos. Tempimas yra svarbus neuronų NAV raiškos veiksnys (40), o kardiomiocitų atsakas į tempimą yra NAV aktyvumo slopinimas (41). Iki šiol nėra aišku, ar stebimas NAV signalų teigiamas poveikis, pasireiškiantis didesniu sienelių įtempimu (pvz., MI atveju), atsiranda dėl tiesioginės NAV funkcijos, ar tai tempimo sukelta NAV sekrecija skatina neuronų KEAV raišką autokrininiu ar parakrininiu būdu, taip skatindama naujų kraujagyslių susiformavimą ir širdies funkcijos padidėjimą.

3. METODIKA

3.1. Žiurkių naujagimių skilvelių miocitų (ŽNSM) kultūros

Visi bandymai su gyvūnais buvo patvirtinti vietos ir valstybinio Gyvūnų tyrimo etikos komiteto (Aachen RWTH Universiteto ligoninė, Vokietija, TV-No. 10596 A). ŽNSM buvo atskirti ir auginami Simpson ir kt. anksčiau aprašytu būdu su nedideliais pakeitimais pagal Zobel ir kt. (42-44) protokolą. Atlikus stuburo slankstelių dislokaciją, paimtos 1-3 dienų amžiaus Sprague–Dawley žiurkių širdys, neatsižvelgiant į žiurkių lytį (Charles River, Sulzfeld, Vokietija) ir suskaidytos paveikiant kolagenaze II (1,0 mg/ml, 280 U/mg, C2-22; Biochrom, Berlynas, Vokietija), tripsinu (0,5 mg/ml, trypsin 1:250, L-11-002; PPA, Colbe, Vokietija) ir 1 proc. penicilino ir streptomicino tirpalu (P-4458; Sigma-Aldrich, Steinheim, Vokietija). Miocitai buvo atskirti nuo fibroblastų, naudojant Percoll tankio gradientą (P-1644; Sigma-Aldrich). Miocitai buvo išsėti į šešių duobučių plokšteles (indus) (140685; Nunc, Schwerte, Vokietija), esant 3×10^5 ląstelių tankiui duobutėje. Ląstelės buvo auginamos DMEM/Ham's F-12 + L-glutamino terpėje (FG-4815; Biochrom) su 10 proc. arklių serumu (B15-021; PPA) ir 5 proc. jaučio embriono kraujo serumu (A-15-101; PPA). Po 24 val. serumo terpė buvo pašalinta, ląstelės praplautos ir toliau laikomos DMEM/Ham's F-12 + L-glutamino terpėje be serumo.

Žmogaus vainikinės arterijos endotelio ląstelės (ŽVAEL) buvo užaugintos endotelio ląstelių augimo terpėje (C-22020; PromoCell GmbH, Heidelberg, Vokietija) prieš pradedant bandymus su NŽSM kondicionuota terpe. „PromoCell" naudotas žmogaus ląstelių kultūrų izoliavimo audinys paimtas iš donorų, pasirašiusių asmens informavimo ir sutikimo formą (formą pasirašė patys donorai, jų įgalioti atstovai ar juridiniai atstovai), kurioje išsamiai aprašytas audinio aukojimo tikslas ir audinio apdorojimo procedūra.

3.2. Ikislenkstinė elektrinė stimuliacija (IES)

Praėjus trims dienoms po ląstelių izoliacijos, terpė su serumu buvo pakeista terpe be serumo ir NŽSM buvo stimuliuojami 48 val. ikislenkstine ir didelio dažnio bipoliarinės elektrinio lauko stimuliacijos būdu (ADES), naudojant C-PaceEP išorinio stimuliavimo sistemą (IonOptix, Wageningen, Nyderlandai). Impulso stiprumas (0,5 V/cm, impulso trukmė - 1 ms) pasirinktas taip, kad sukurtų elektrinį lauką, gerokai silpnesnį nei slenkstinė reikšmė, sukelianti išaugintų miocitų susitraukimą. Mažiausia stimuliavimo srovė įvairiuose preparatuose buvo 1.0 V/cm. Miocitų kontrakcijos nebuvimas buvo tikrinamas kas 12 val. bandymo metu, tikrinant pasėlius mikroskopu. Buvo pasirinkti dažniai: 5 Hz, 25 Hz, 50 Hz ir 99 Hz. Kontrolei naudotos ląstelės, kurioms netaikyta didelio dažnio elektrinė stimuliacija.

3.3. ELISA analizė

Atliekant KEAV imunofermentinę analizę (ELISA), ląstelių skaičius kiekvienoje duobutėje buvo normalizuotas iki 300 000 ir atlikta IES analizė, naudojant dažnius nuo 5 Hz iki 25 Hz, 50 Hz ir 99 Hz. Kondicionuota terpė buvo surinkta po 0, 24 ir 48 val. po IES ir ląstelių kultūros baltymas buvo koncentruotas „Sartorius“ centrifugavimo kolonėlėse (VivaSpin) (Göttingen, Vokietija). Pavyzdžių analizė atlikta su „R&D Systems“ KEAV ELISA rinkiniu (Minneapolis, MN, JAV), normalizavus baltymų kiekius pagal gamintojo instrukcijas. Baltymų kiekis nustatytas naudojant „Thermo Scientific“ prietaisą NanoDrop 1000 (Wilmington, DE, JAV).

3.4. *Western Blot* metodas

KEAV ir KDR (kenazės domeno įterpimo receptoriaus) normalizuotų baltymų kiekių tyrimai atlikti *Western Blot* (WB) metodu. WB analizei atlikti ląstelės buvo homogenizuotos lizės buferiniame tirpale, turinčiame 20 mM Tris-HCl (pH 8,0; #9090.2; Carl-Roth, Karlsruhe, Vokietija), 1 proc. nejoninio detergento Nonidet P-40 (#74.385; Merck, Darmstadt, Vokietija) ir 10% glicerolio (#50405-1; Biomol, Hamburg, Vokietija). Ląstelių lizatai nuskaidrinti 20 min. centrifuguojant 17 000×*g*. Ekstraktai (100 µg) separuoti atliekant SDS-poliakrilamido gelio (10 proc.) (SDS #51430-2, Biomol/ polyacrylamide gel #3029.1; Carl-Roth) elektroforezę ir perkelti ant polivinilideno difluorido membranos (#RPN303F; GE-Healthcare Life Sciences, Freiburg, Vokietija). Membrana per naktį blokuota su 5 proc. galvijų serumo albuminu (#01400-1; Biomol) buferiniame fosfato druskų tirpale (#L-1825; Biochrom), turinčiame 0,1 proc. Tween 20 (PBST; #TW0020; Rockland, ME, JAV). Triušių anti-GAPDH pirminiai antikūnai (1:1 000; #2118; Cell Signaling, Danvers, MA, JAV), triušių anti-KEAV (1:150; 19003-1-AP; Acris Antibodies, Herford, Vokietija) ir triušių anti-KDR (200 µg/ml, 1:100; sc-315; Santa Cruz, Heidelberg, Vokietija) buvo inkubuojami per naktį. Blotai buvo perplauti 3 kartus su buferiniu fosfato druskų

tirpalu, turinčiu 0,1 proc. Tween 20, ir 1 val. inkubuoti su anti-triušio antikūnais, gautais iš ožkos ir konjuguotais su krienų peroksidaze (GE Healthcare). Galiausiai, juostelės buvo išryškintos naudojant ECL Advance Western Blotting Detection Kit rinkinį (GE Healthcare Life Sciences) ir „Fujifilm“ (Tokyo, Japonija) pažangų liuminescencinį vaizdo analizatorių LAS-3000. Atitinkama juostelių densitometrija atlikta „Fujifilm“ kompiuterine programa Multi-Gauche V3.0.

3.5. Antikūnai

Ląstelės buvo užfiksuotos 4 proc. formaldehido tirpale (8.187.15; KMF, Lohmar, Vokietija), permeabilizuotos Triton X-100 (50800-1; Biomol) ir blokuojamos 1 proc. galvijų serumo albumino tirpale (01400-1; Biomol). Ląstelės buvo per naktį inkubuojamos su pirminiais antikūnais, (1:150) triušių anti-KEAV (200 µg/ml, 1:150; sc-507; Santa Cruz) ir pelių anti-Trop T-C (200 µg/ ml; sc-20025; Santa Cruz) 4°C temperatūroje, o po to 2 val. inkubuojamos su iš ožkos gautais anti-triušio (1:100) ir iš ožkos gautais anti-pelių (1:100) antriniais antikūnais kartu su Alexa Fluor 488 (KEAV) (1 mg/ml; A11008; Invitrogen) arba Alexa Fluor 647 (Trop T-C) (1 mg/ml; A20990; Invitrogen). Branduoliai buvo užpilti 4‘,6-diamidino-2-fenilindolo dihidrochloridu (10236276001; Roche Mannheim, Vokietija). Fluorescenciniai signalai vizualizuoti Axiovert 200M mikroskopu, naudojant „Zeiss“ (Jena, Vokietija) kompiuterinę programą AxioVision Rel. 4.5.

Pirminiai antikūnai, naudoti taikant *Western Blot* metodą: triušių anti-GAPDH (1:1,000; #2118; Cell Signaling), triušių anti-KEAV (1:150; 19003-1-AP; Acris Antibodies), triušių anti-KDR (200 µg/ml, 1:100; sc-315; Santa Cruz).

3.6. RNR paruošimas, kopijinės DNR (kDNR) pirmos grandies sintezė ir realaus laiko kiekybinė atvirkštinės transkripcijos PGR

Bendroji RNR išskirta iš ŽNSM Qiagen RNeasy Mini Kit rinkiniu (Venio, Nyderlandai), laikantis gamintojo instrukcijų. 1 µg RNR atvirkštinė transkripcija atlikta „Fermentas“ kDNR pirmos grandies sintezės rinkiniu, naudojant atsitiktinius heksamerus (#K1622; Fermentas GmbH, St. Leon- Rot, Vokietija).

3.7. Realaus laiko kiekybinė atvirkštinė transkripcija - PGR. Realaus laiko kiekybinės atvirkštinės transkripcijos - PGR pradmenys ir zondai

Realaus laiko polimerazinė grandininė reakcija (PGR) buvo vykdoma 96 duobučių PGR plokštelėse, taikant pirmiau aprašytą Step-One plus sekos aptikimo sistemą (ABI) (9). Duomenys buvo renkami spektrinės kompensacijos metodu, naudojant „Applied Biosystems“ kompiuterinę programą SDS 1.2.3 (Applied Biosystems, Foster City, CA, JAV).

PGR pradmenys ir fluorogeniniai zondai tiksliniams genams ir endogeninei kontrolei buvo perkami kaip užsakomieji tyrimai (Applied Biosystems). Endogeninės kontrolės ir tikslinių genų tyrimų numeracija: Rn00560865_m1 (b-2 microglobulin), Rn01533872_m1 (Ngfb), Rn00561661_m1 (Nppa). Tyrimų numeriai: Rn01511602_m1 (KEAV), Rn00564986_m1 (KDR), Rn00560865_m1 (b-2 microglobulin).

3.8. Žmogaus vainikinės arterijos endotelio ląstelių proliferacijos tyrimas

Siekdami nustatyti ŽVAEL proliferaciją dėl NŽSM, kuriems taikyta IES, išskiriamo bioaktyvaus KEAV, mes naudojome „PromoCell“ kolorimetrinės analizės rinkinį (WST-8). WST-8 yra ląstelių dehidrogenazių biologiškai redukuojamas iki oranžinės spalvos formazano junginio, kuris tirpsta ląstelių pasėlių terpėje. Kadangi formazano kiekis yra tiesiogiai proporcingas gyvų ląstelių skaičiui, šis metodas leidžia tiksliai nustatyti gyvybingų ląstelių skaičių ląstelių pasėlyje.

ŽVAEL buvo inkubuojamos 48 val. NŽSM kondicionuotoje terpėje. 25 Hz ir 5 Hz dažniai buvo analizuoti, kadangi dominuojantis KEAV indukcijos poveikis stebėtas taikant 25 Hz dažnį (lyginant su 5 Hz dažniu ir kontrole).

3.9. Pacientų su DKMP atranka, mechaninis tempimas ir Tunel analizės metodas

Pacientams su DKMP (n 1⁄4 5, amžiaus vidurkis 50,3 ± 3,5 m.), kuriems pasireiškia ryški kairiojo skilvelio disfunkcija (KSIF < 30%, Niujorko širdies asociacijos (NYHA) II funkcinė klasė), buvo taikoma IA terapija penkias dienas iš eilės. Po paskutinio IA seanso visiems pacientams buvo skirtas polikloninis IgG (0,5 g/kg) koncentracijai kraujo plazmoje atstatyti (45). Tokie patys atitinkamos koncentracijos poliklonіniai IgG buvo naudoti ląstelių pasėlių kontrolės tikslais ir yra nurodyti atitinkamose diagramose kaip sveiki kontroliniai IgG (SK-IgG). Visiems pacientams su DKMP yra buvęs širdies nepakankamumas prieš vienerius metus ar daugiau metų ir jie visi ne trumpiau nei 6 mėn. stabiliai vartojo geriamuosius vaistus, įskaitant angiotenziną konvertuojančių fermentų inhibitorius ar angiotenzino receptorių blokatorius, diuretikus, aldosterono antagonistus ir **β**-blokatorius. Pacientai, kuriems atlikus angiografiją buvo nustatyta išeminė liga, į tyrimą buvo neįtraukti. IA terapijos metu pacientams buvo skiriamos A baltymas Immunoadsorba (Fresenius Medical Care AG, Bad Hombug, Vokietija). IA eliuentas (KE) su išplautu IgG buvo surinktas ir paruoštas kituose šaltiniuose aprašytiems ląstelių pasėlių bandymams (22). KE baltymo koncentracijai nustatyti naudotas prietaisas Nanodrop ND-1000 (PeqLab, Erlangen, Vokietija). KE alikvotinės dalys iki naudojimo buvo laikomos 80ºC temperatūroje. Pacientų su DKMP IgG koncentracija serume buvo stebima ir nustatytas IgG koncentracijos redukcijos vidurkis buvo 91,7 proc., matuojant penktą dieną po paskutinio IA terapijos seanso. Pacientų su išemine kardiomiopatija (IKMP)

(n l⁄4 7) išmetimo frakcijos, KS dydžiai ir NYHA funkcinės klasės parametrai buvo panašūs į pacientų su DKM parametrus (duomenys nepateikti).

Mechaniniam tempimui atlikti NŽKM buvo inkubuojami ant kolagenu dengtų silikoninių membranų ir atliktas prisitvirtinusių miocitų statinis tempimas, palaipsniui didinant tempimą, kaip aprašyta pirmiau (46). Tempimas buvo laipsniškai didinamas kas 24 val. nuo 3 proc. iki 13 proc. (3, 7, 13 proc. laipsniais).

TUNEL analizė buvo atlikta „APO-BrdU“ TUNEL analizės rinkiniu pagal gamintojo instrukcijas. Ląstelių apoptozė buvo nustatyta suskaičiavus TUNEL-teigiamų branduolių skaičių, išreikštą procentu nuo visų suskaičiuotų ląstelių (47).

3.10 Statistinė analizė

Visos reikšmės išreikštos vidurkiu ± SN. Duomenys apdoroti naudojant statistinį duomenų analizės paketą SPSS 14.0 for Windows (IBM, Somers, NY, JAV). Statistinis skirtumų reikšmingumas įvertintas pagal porinį Stjudento t kriterijų ir vieno faktoriaus analizę ANOVA, atliekant post-hoc mažiausio reikšmino skirtumo palyginimus tarp įvairių grupių. Rezultatas laikomas statistiškai reikšmingu, kai $p < 0,05$.

4. REZULTATAI

4.1. IES padidina žiurkių naujagimių KEAV ekspresiją ir sekreciją

Siekiant nustatyti IES poveikį kardiomiocitų KEAV ekspresijai, ŽNSM ląstelių kultūra buvo stimuliuojama 48 val. 5 Hz, 25 Hz, 50 Hz ir 99 Hz dažniais. KEAV baltymų kiekis ląstelių pasėlių terpėje matuotas po 24 ir 48 stimuliavimo valandų. Didelio dažnio elektrinė stimuliacija (DDES) sukėlė KEAV ekspresijos padidėjimą tiek citoplazmoje, tiek ir ląstelių išorėje. IES dominuojantis efektas stebėtas taikant 25 Hz dažnį, kai KEAV baltymų kiekis citoplazmoje padidėjo 1,8 karto. KEAV sekcija į ląstelės išorę taip pat reikšmingai padidėjo, sudarant beveik varpo formos dozės-efekto kreivę, kurios aukščiausias taškas pasiektas taikant 25 Hz dažnį. DDES poveikis KEAV ekspresijai išliko ne mažiau kaip 48 val., nepriklausomai nuo stimuliacijos dažnio. Skirtingi KEAV baltymo kiekiai matomi imunocitofluorescenciniuose vaizduose.

Mes atlikome ląstelių lizatų KEAV informacinės RNR (iRNR) turinio analizę, kad įvertintume padidėjusios KEAV genų ekspresijos santykinį poveikį padidėjusiai KEAV baltymų raiškai.

4.2. KEAV iRNR ir KEAV receptoriaus pokyčiai DDES ŽNSM metu

DDES sukėlė ženklų KEAV iRNR sumažėjimą, kuris buvo statistiškai reikšmingas taikant 25 Hz, 50 Hz, 99 Hz dažnius ir maksimalus esant 25 Hz dažniui. Kadangi

taip galėjo atsitikti dėl neigiamo grįžtamojo poveikio atsako į padidėjusius KEAV baltymo kiekius tiek citoplazmoje, tiek ir ląstelės išorėje, mes išmatavome membranos KEAV receptoriaus (KDR) raišką tiek baltymų, tiek ir iRNR atžvilgiu. IES reikšmingai sumažino KDR receptoriaus baltymų raišką esant 25 Hz dažniui ir nereikšmingai - taikant 50 Hz ir 99 Hz dažnius, tuo tarpu kai KDR receptoriaus iRNR reikšmingai padidėjo IES metu taikant 25 Hz, 50 Hz ir 99 Hz dažnius. Pažymėtina, kad KEAV iRNR ekspresijos ir KDR baltymų bei genų ekspresijos santykinio sumažėjimo priklausomybė nuo dažnių buvo tokia pati, kaip ir KEAV baltymų indukcijos atveju, t.y. reikšmingiausias dydis stebėtas taikant 25 Hz dažnį.

4.3. IES indukuota KEAV ekspresija yra biologiškai aktyvi ir skatina žmogaus vainikinių arterijų endotelio ląstelių proliferaciją

Siekiant nustatyti, ar stebimas IES indukuotas KEAV veiksmingai skatina endotelio ląstelių proliferaciją, ŽVAEL buvo inkubuojamos NŽSM kondicionuotoje terpėje (IES trukmė 48 val., atitinkamai taikant 5 Hz, 25 Hz, 50 Hz ir 99 Hz dažnius). Tyrimo metu stebėta, kad terpėje, kondicionuotoje 25 Hz dažnio IES, ŽVAEL augimas padidėjo, o 5 Hz dažnio IES kondicionuotoje terpėje reikšmingo atsakomojo ŽVAEL augimo *in vitro* nestebėta.

4.4. Pacientų su DKMP padidėjusi KEAV koncentracija serume sumažėja po IA terapijos

Kadangi padidėjęs širdies sienelės tempimas gali turėti įtakos KEAV raiškai, pacientų, sergančių DKMP, KEAV koncentracija serume buvo palyginta su pacientais, kuriems diagnozuota išeminė kardiomiopatija (IKMP). Reikšmingų kairiojo skilvelio išstūmimo frakcijos bei KS galinio sistolinio ir galinio diastolinio diametrų skirtumų tarp dviejų grupių nenustatyta (duomenys nepateikti). Nedidelėje mūsų pacientų su DKMP grupėje pradinė KEAV koncentracija serume buvo žymiai didesnė, lyginant su pacientais IKMP grupėje. Po keturių savaičių IA terapijos buvo stebėtas reikšmingas KEAV koncentracijos serume sumažėjimas pacientų DKMP grupėje, lyginant su pradine koncentracija. Šiame etape KEAV koncentracija serume abejose grupėse buvo beveik vienoda (lyginant DKMP grupę su IKMP grupe), o reikšmingų aukščiau minėtų echokardiografinių skirtumų nenustatyta.

4.5. Pirminis ŽNKM veikimas DKMP-IgG skatina KEAV raišką

Tirdami ląstelių kultūrą, pirmiausia buvo siekiama išsiaiškinti su DKMP-IgG doze susijusią ŽNKM reakciją apoptozės atžvilgiu. Pastebėjome, kad apoptozės lygis šiek tiek padidėjo, lyginant su pradiniu lygiu, kai DKM-IgG koncentracija buvo 500 ng/ml. Taigi, siekiant išvengti šalutinio poveikio dėl ląstelių žūties, visi tolimesni bandymai buvo vykdomi naudojant DKMP-IgG, kurių koncentracija neviršijo 100 ng/ml. Esant tokiai koncentracijai ŽNKM apoptozė nepadidėja, tačiau reikšmingai pa-

didėja KEAV raiška iRNR ir baltymo lygmenyse. DKMP-IgG koncentracijai viršijus 500 ng/ml, stebėtas atvirkštinis poveikis, kai KEAV koncentracija sumažėjo iki kontrolinių ląstelių ribų galimai dėl padidėjusios ląstelių žūties.

Taikant ELISA metodą, galima ištirti KEAV sekreciją ląstelių išorėje priklausomai nuo laiko. Kai DKMP-IgG koncentracija yra 100 ng/ml, KEAV sekrecijos padidėjimas tampa statistiškai reikšmingas po 24 val., lyginant su 0 val., o po 48 val. šis skirtumas yra reikšmingas lyginant su kontrolinėmis ląstelėmis, kurios buvo veikiamos SK-IgG. ŽNKM, kurie buvo veikiami 100 ng/ml koncentracijos sveikų donorų IgG, po 48 val. intervalo nerodė jokių reikšmingų KEAV sekrecijos pokyčių.

4.6. KEAV raiška ŽNKM padidėja ne dėl susitraukimo dažnio, bet dėl mechaninio tempimo

Sergant DKMP, padidėja širdies susitraukimų dažnis ir mechaninė perkrova. Todėl mes tyrėme, kokią įtaką šie du parametrai daro KEAV ekspresijai ląstelių lygmenyje. Kai DKMP-IgG koncentracija 100 ng/ml, mikroskopu stebimas ŽNKM susitraukimo dažnio padidėjimas. Didinant DKMP-IgG koncentraciją, susitraukimų dažnis mažėja, tikėtina, dėl ląstelių pažeidimo ir galutinės apoptozės, o kontrolinės ląstelės, kurios buvo inkubuojamos su tokios pačios koncentracijos sveikų donorų IgG, lieka nepažeistos. ŽNKM, kurie prieš tai buvo paveikti selektyviu beta1-adrenerginių receptorių antagonistu - metoprololiu, susitraukimų dažnis sumažėjo be jokio poveikio KEAV ekspresijai. Ir priešingai, mechaninis tempimas padidina ŽNKM KEAV raišką. Inkubuojant ištemptas ląsteles su 100 ng/ml koncentracijos DKMP-IgG, pasiektas papildomas KEAV indukcijos efektas (KEAV baltymo raiška; kontrolė: 1 ± 0,0, palyginus su 13% tempimu: 1,75 ± 0,15 ($P < 0,05$), palyginus su 13% tempimu þ 100 ng/ml DKM-IgG: 1,95 ± 0,23).

4.7. Nereguliarus stimuliavimas padidina skilvelių miocitų NAV ekspresiją

Buvo analizuota NAV iRNR raiška kardiomiocituose, didinat stimuliacijos dažnį (0 Hz, 5 Hz ir 50 Hz) ir stimuliuojant nereguliariai (5, 25 ir 50 proc. SN). Taikant pastovų 5 Hz dažnį, stebėtas nedidelis, statistiškai nereikšmingas NAV ekspresijos padidėjimas, esant nedideliam nereguliarumo laipsniui (5 proc. SN). NAV iRNR ekspresijos padidėjimas tapo reikšmingas, kai nereguliarumo laipsnis pasiekė 25 proc. SN. Kai nereguliari stimuliacija pasiekė 50 proc. SN, NAV iRNR raiška vėl sumažėjo iki kontrolės lygio. Tuo tarpu esant pastoviam 50 Hz dažniui, NAV iRNR raiška reikšmingai sumažėjo, nepriklausomai nuo stimuliavimo nereguliarumo. Stimuliuojant 50 Hz dažniu ir keičiant nereguliarumo laipsnį iki 50 proc. SN, jokio poveikio nenustatyta.

Siekiant įvertinti NAV sekreciją ląstelių išorėje, buvo atlikti tyrimai ELISA metodu. Didžiausias poveikis buvo stebėtas taikant 5 Hz dažnį su 25 proc. SN. Kita

vertus, taikant 50 Hz ADES, NAV lygis reikšmingai sumažėja. Šiam poveikiui nereguliarumas įtakos neturėjo. ELISA metodu gautus duomenis patvirtina bandymai *Western Blot* metodu. Neritmiška stimuliacija reikšmingai padidina NAV baltymų kiekį kardiomiocituose.

4.8. Atliekant nereguliarią stimuliaciją, NAV padidėjimas grįžta į kontrolinį lygį stimuliaciją nutraukus

Siekiant nustatyti, ar neritmiškos stimuliacijos poveikis NAV ekspresijai kardiomiocituose išlieka nutraukus nereguliarią stimuliaciją, buvo atlikti tyrimai, keičiant stimuliavimo programą. Po pradinės neritmiškos stimuliacijos, kurios metu buvo taikytas 5 Hz dažnis su 25 proc. SN pirmas 24 val., vėliau stimuliacija buvo tęsiama ne be 25 proc. SN. Tyrimas parodė, kad nereguliarios stimuliacijos sukeltas pradinis NAV ekspresijos padidėjimas (per pirmas 24 val.) beveik grįžo iki pradinio kontrolinio lygmens nutraukus nereguliarią stimuliaciją.

4.9. NAV koncentracijos padidėjimas dėl neritmiško stimuliavimo yra pakankamas simpatinės sistemos aksonų ataugimui skatinti

Siekiant nustatyti, ar kardiomiocitų NAV ekspresijos ir sekrecijos padidėjimas, pasiektas atliekant neritmišką stimuliavimą, yra pakankamas, kad skatintų simpatinės sistemos aksonų ataugimą, pirminiai simpatinės sistemos neuronų pasėliai buvo inkubuojami 48 val. kardiomiocitų ląstelėmis kondicionuotoje terpėje, paimtoje po 48 val. elektrinės stimuliacijos. Neritmiško stimuliavimo sukelta didesnė NAV baltymo koncentracija reikšmingai padidino aksonų ataugimą, nustatytą atliekant baltymo GAP-43 (neuronų augimo markerio) raiškos bandymus imunocitofluorescenciniu ir *Western Blot* metodu.

4.10. Mechaninis tempimas padidina neuronų KEAV raišką

SSN tempimas buvo laipsniškai didinamas, kaip aprašyta skyriuje „Metodika“. KEAV genų raiška reikšmingai padidėjo po 72 val. tempimo. Bandymai *Western Blot* metodu patvirtino, kad kartu su KEAV iRNR koncentracija padidėja ir KEAV baltymų raiška. Naudodami didelio jautrumo tyrimų rinkinius ELISA, buvo ištirta KEAV sekrecija SSN ląstelių išorėje. Šie bandymai parodė, kad tempimo indukuota neuronų KEAV raiška priklauso nuo laiko ir dozės. Per pirmas 24 val. tempiant 3 proc., KEAV kiekis liko pradiniame lygmenyje. Nuo 24 val. iki 48 val. tempiant 7 proc., stebėtas nereikšmingas KEAV ekspresijos padidėjimas, kuris tapo statistiškai reikšmingu po 72 val. 13 proc. tempimo (duomenys nepateikiami).

4.11. Tempimo sukelta neuronų KEAV sekrecija yra biologiškai aktyvi

Siekiant nustatyti, ar tempimo sukelta neuronų KEAV sekrecija yra biologiškai aktyvi, buvo atlikta ląstelių gyvybingumo bandymus su ŽVAEL „Metodikos“ skyriuje aprašytu būdu. Kondicionuotoje ištemptų neuronų terpėje buvo stebėtas ženkliai didesnis ląstelių gyvybingumas ŽVAEL, lyginant su ŽVAEL inkubuotų neuronų, kuriems tempimas netaikytas, terpėje.

5. REZULTATŲ APTARIMAS

Pagrindiniai mūsų tyrimo rezultatai yra: a) IES reikšmingai padidina išaugintų ŽNSM KEAV baltymų raišką, b) dominuojantis IES poveikis KEAV raiškai stebėtas taikant 25 Hz dažnį, c) IES indukuota ŽNSM KEAV sekrecija yra biologiškai aktyvi ir skatina ŽVAEL proliferaciją *in vitro*.

Stipriausias IES poveikis KEAV raiškai stebėtas taikant 25 Hz dažnį, kuomet ekstraląstelinė KEAV baltymų koncentracija padidėjo beveik dvigubai. KEAV ekspresijos priklausomybė nuo dažnio, atliekant IES *in vitro*, buvo aprašyta anksčiau, atliekant kitų ląstelių junginių bandymus, t.y. taikant 50 Hz dažnį skeleto raumenų ląstelėms ir 24 Hz dažnį lygiųjų raumenų ląstelėms (48). Tikslus mechanizmas, kaip DDES padidina ląstelių baltymų raišką, nėra aiškus. Miokardo ar širdies neuronų ląstelėse funkcinis elektrostimuliacijos dirginamasis poveikis (pvz., raumenų ląstelių susitraukimas arba neuronų iškrova) priklauso nuo membranos depoliarizacijos, kurią, savo ruožtu, lemia refrakterinis laikotarpis. Todėl aukštesnių dažnių, kurių refrakterinis laikotarpis yra trumpesnis, poveikis širdies neuronų ląstelėms paprastai būna stipresnis. IES trofinis poveikis taip pat buvo aprašytas, atliekant simpatinės ir parasimpatinės sistemos neuronų ląstelių bandymus (žr. mūsų grupę). Aukštų dažnių stimuliacija padidino NAV, baltymo NT-3 ir genų ekspresiją, kuri, savo ruožtu, paskatino neuronų ląstelių augimą ir hipertrofiją *in vitro* ir *in vivo* (49). Tačiau, priešingai nei šiame tyrime, minėtas poveikis priklausė nuo neuronų ląstelių depoliarizacijos, kadangi buvo naudojama ikislenkstinė srovė ir poveikį slopino lidokainas (50).

Šiame tyrime ikislenkstinė elektros stimuliacija negalėjo sukelti miokardo depoliarizacijos, todėl dėl skirtingo elektros srovės poveikio galėjo vykti tik ląstelių membranos depoliarizacija.

Ypač atkreiptinas dėmesys, kad IES yra universalaus pobūdžio mechanizmas, galintis padidinti ląstelių augimo veiksnio raišką. Šį teiginį taip pat patvirtina nuo dažnio priklausantis į insuliną panašaus augimo veiksnio–2 baltymų ir genų ekspresijos padidėjimas osteosarkomos ląstelėse (51). Minėtas poveikis neapsiriboja vien tik augimo veiksnių indukcija, kadangi šaltiniuose taip pat yra aprašyta didesnė nuolatinė azoto oksido sintazė IES metu (48).

Teoriškai, IES galėtų padidinti KEAV baltymo stabilumą. Tačiau, Kanno ir kt. atlikti tyrimai parodė, kad elektrinė stimuliacija neturėjo poveikio KEAV iRNR stabilumui. Tai įrodo, kad KEAV iRNR padidėjimas dėl elektrinės stimuliacijos iš esmės vyksta transkripciniame lygmenyje. Be to, atlikus stimuliaciją nenusistovėjusiu elektriniu lauku (2 val., plg. su 24 val.) gautas beveik identiškas KEAV iRNR lygis, kas rodo, kad pradinio IES sukelto iRNR padidėjimo pakako indukuoti ilgalaikį KEAV baltymų sintezės padidėjimą. Šią išvadą taip pat patvirtina pastebėjimai, kad KEAV baltymų ekspresija pradeda didėti vėliau, po 12 val. nuo IES pradžios, o maksimalus padidėjimas stebėtas po 48 val. (4).

Šiame tyrime KEAV iRNR lygis sumažėjo po 48 val. IES, nors KEAV baltymo koncentracija padidėjo. Kadangi KDR receptoriaus iRNR ir baltymai sumažėjo, galėjo suveikti padidėjusios KEAV baltymų koncentracijos neigiamo grįžtamojo ryšio KEAV iRNR ekspresijai mechanizmas (per KDR kiekio sumažėjimą). Iš tiesų, Hang su kolegomis jau nustatė, kad KEAV iRNR raiška palaipsniui mažėja tęsiant elektrinę stimuliaciją ilgą laiką ir kad KEAV indukuota angiogenezė gali priklausyti nuo neigiamo grįžtamojo ryšio (52). Taigi, jei riboto IES taikymo sukelto KEAV iRNR padidėjimo pakanka ilgalaikei KEAV baltymų raiškai indukuoti, reflektorinis KEAV iRNR raiškos mažėjimas gali prasidėti jau per 48 val. stimuliacijos laikotarpį ir todėl bendras iRNR lygis bus žemesnis nei kontrolinėje grupėje.

Pažymėtina, kad IES indukuoto KEAV padidėjimo antrinis neigiamas grįžtamasis ryšys gali neutralizuoti teigiamą IES poveikį terapinei angiogenezei. Kadangi pagal galimą klinikinio pritaikymo scenarijų IES gali būti vykdoma tik trumpą laiką (pvz., intrakardialiniais kateteriais), angiogenezės indukcijos gali pakakti klinikiniu požiūriu reikšmingam revaskuliarizacijos poveikiui pasiekti. Šią hipotezę patvirtina ir mūsų atliktas tyrimas, kurio metu trumpalaikė 48 val. IES sukėlė KEAV baltymų padidėjimą, kurio pakako ląstelių augimui indukuoti *in vitro*.

Kai kurių tyrimų rezultatai įtikinamai rodo teigiamą sistemingai taikomos elektrinio lauko stimuliacijos poveikį įvairių ląstelių pasėliams ir gyvūnų modeliams. Shafy ir kt. atliktas tyrimas parodė, kad sisteminga elektrinė stimuliacija širdies resinchronizacijos terapijos prietaisu padidina autologinių mioblastų, įšvirkštų į avies modelio miokardo infarkto pažeistas sritis, diferenciaciją. Autoriai įrodė, kad šios technikos taikymas pagerino miokardo funkciją (53). Haneef ir kt. pastarųjų metų tyrimai taip pat pademonstravo, kad kamieninių ląstelių elektrostimuliacija yra saugus ir veiksmingas metodas ląstelių išgyvenamumo ir diferenciacijos į širdies ląsteles požiūriu (54). Kalbant apie KEAV vaidmenį, Spadaccio ir bendradarbiai atskleidė, kad nuolatinė elektrinė stimuliacija miokardo infarkto *in vivo* modelyje pagerino miokardo funkciją ir tuo pačiu pagerėjo angiogenezė dėl endotelio progenitorinių ląstelių migracijos ir KEAV gamybos (43). Šie nauji tyrimai skatina miokardo audinių inžinerijos tyrimus, orientuotus į naujus išeminės širdies ligos gydymo metodus. Mūsų tyrimas visiškai atitinka minėtų tyrimų rezultatus. Be to, naujų širdies lygų

gydymo metodų prasme, tikėtina, kad galimybė atlikti IES yra saugesnė, siekiant išvengti pašalinio poveikio, tokio kaip aritmija.

Su autoimunine sistema susijusių angiogeninių signalų perdavimas yra aprašytas, pavyzdžiui, *lupus erythematodes systemicus* arba *myasthenia gravis* atvejais (55, 56). Pastaruoju atveju, autoriai nustatė pacientų, sergančių *myasthenia gravis*, santykinę teigiamą KEAV koncentracijos koreliaciją, lyginant su sveikais kontrolinės grupės tiriamaisiais. Be to, atliekant vėžio tyrimus, žinoma, kad proangiogeniniai ir antiangiogeniniai veiksniai daug lemia auglių progresavimui ir ligos plitimui (57, 58). KEAV yra pagrindinis stiprus angiogeninis veiksnys, kuris atsako už naujų kraujagyslių formavimąsi. KEAV indukcija vėžio ląstelėse iki šiol nėra visiškai aiški. Lombardi su kolegomis įtikinamai nustatė, kad krūties vėžio IgG padidino KEAV ekspresiją auglio ląstelėse. Tokį atsaką paskatino muskarininių receptorių siunčiami signalai. Šie rezultatai ir mūsų tyrimo rezultatai akivaizdžiai patvirtina, kad antikūnai yra stiprūs augimo veiksnių, ypač KEAV, induktoriai įvairių ligų ir patologinių aplinkybių atvejais (59).

KEAV ir du jo receptoriai KEAVR-1 ir KEAVR-2 vaidiną pagrindinį vaidmenį angiogenezėje fiziologinėmis ar patologinėmis aplinkybėmis ir dalyvauja neuronų ir miokardo vystymesi bei morfogenezėje (60). Tyrimai su gyvūnų modeliais atskleidė, kad net ir nedideli KEAV koncentracijos sutrikimai neigiamai veikia širdies kraujagyslių vystymąsi ir embrionų išgyvenamumą (61,62). Nenormalios KEAV būsenos gali sukelti kardiomiocitų hipertrofiją, dėl kurios gali prasidėti ar progresuoti širdies remodeliacija (11,12). Be to, labai svarbu yra tai, kad kardiomiocitų išskiriamas KEAV parakrininiu būdu skatina širdies fibroblastų proliferaciją (13). Patologinė fibroblastų proliferacija gali paskatinti miokardo fibrozės progresavimą. Dėl mikrocirkuliacijos sutrikimų sumažėja deguonies slėgis ir tokiu būdu vėl skatinama KEAV indukcija. Šis ratas gali sąlygoti nevaldomos ir nefiziologinės KEAV gamybos sukeltos patologinės remodeliacijos progresavimą. Tokias prielaidas patvirtina ir tai, kad nustatyta, jog mechaninė perkrova, kuri yra plačiai paplitęs DKMP stimulas, taip pat padidina kardiomiocitų KEAV raišką, kurią autoantikūnai toliau didina.

Šis tyrimas praplečia žinias apie galimą sąryšį tarp autoimuninių signalų DKMP atveju ir KEAV indukcijos kardiomiocituose. Šis metodas gali padėti kuriant naujus farmakologinius metodus miokardo remodeliacijai DKMP atvejais moduliuoti molekuliniu pagrindu.

NAV ne tik yra pagrindinis simpatinės sistemos neuronų išgyvenamumo ir diferenciacijos veiksnys, bet taip pat atlieka ir kardiomiocitų išgyvenamumą stiprinančio veiksnio, kuris reguliuoja apoptozę, funkciją (63) bei yra labai svarbus širdies funkcijos atsistatymui po miokardo infarkto (64). NAV reguliacija taip pat yra susijusi su nervinių ataugų augimu ir staigia širdies mirtimi (65). Nors NAV neabejotinai dalyvauja širdies neurofiziologijoje, mechanizmas, kaip širdies NAV raiška padidėja ar sumažėja, kol kas yra sunkiai paaiškinama. Taip pat žinoma, kad mechaninis tempimas, universalus širdies audinio stimulas, skatina hipertrofiją ir NAV sumažėjimą

kardiomiocituose Cn(kalcineurino)/NFAT (aktyvuotų T ląstelių branduolių faktorių) keliu (66).

Tempimas širdies ir kraujagyslių sistemoje vykdomas skirtingomis formomis ir laipsniais (ciklišKas tempimas, statinis pradinio lygio tempimas ir tempimas dėl didėjančios šlyties jėgos). MI ar arterijų hipertenzijos metu intrakardinės ląstelės vis labiau tempiamos, lyginant su pradiniu lygiu. Kalbant apie su tempimu susijusią neurotrofinę reguliaciją, kardiomiocitai NAV raišką mažina, o SSN atsakas į tempimą yra įvairių neurotrofinų, įskaitant NAV, kiekio padidėjimas (40,41). Šiame tyrime mes nustatėme, kad SSN tempimas vyksta kartu su KEAV indukcija ir priklauso nuo laiko ir dozės. Todėl NAV signalai vaidina svarbų vaidmenį, tuo tarpu neurotrofinių veiksnių NT-3 ir GDNF įtakos reikšmingumą neuronų KEAV indukcijai galima atmesti. Be to, ŽVAEL gyvybingumo bandymai patvirtino, kad mechaninio tempimo sąlygomis išskiriama neuronų KEAV koncentracija yra biologiškai aktyvi. Vienas kardiomiocitas atsako į tempimą padidėjusiu KEAV kiekiu ir tai yra vienas ląstelių šaltinis (29). Kadangi širdies autonominė nervų sistema dalyvauja reguliuojant įvairius fiziologinius ir patologinius reiškinius, žinojimas, kad SSN taip pat gali gaminti ir išskirti KEAV yra neabejotinai svarbus (34).

Simpatinės nervų sistemos reguliavimo mechanizmai veikia širdies ir kraujagyslių sistemą per postganglinius simpatinės sistemos neuronus, kurie inervuoja kraujagysles ir širdį. Kraujagyslių simpatinės inervacijos poveikis kraujo spaudimui ir kraujotakai yra labai didelis. Todėl kraujagyslių simpatinės inervacijos pokyčiai siejami su širdies ir kraujagyslių ligų išsivystymu ir buvimu (67). Teigiama, kad KEAV ir jo receptoriai skatina kraujagyslių simpatinę inervaciją (68, 69). Taigi, KEAV ne tik reguliuoja kolateralinių kraujagyslių, kaip stipraus angiogeninio veiksnio hipoksijos ar kitų biocheminių ir biomechaninių jėgų prasme, susidarymą, bet ir daro didelę įtaką širdies ir kraujagyslių reguliavimo sistemų fiziologijai.

NAV yra pagrindinis neurotrofinis faktorius, reguliuojantis SSN išgyvenamumą ir diferenciaciją (70). Pastarųjų metų tyrimai nustatė, kad NAV taip pat yra kardiomiocitų išgyvenamumą gerinantis veiksnys (71). Be to, NAV skatina angiogenezę išeminėse užpakalinėse galūnėse (10) ir gerina širdies funkciją po MI (39). Tikėtina, kad šie pastebėjimai susiję su kryžminiu NAV ir KEAV reguliacijos poveikiu. MI metu intrakardinės ląstelės vis labiau tempiamos. Iš vienos pusės, tempimas slopina NAV ekspresiją kardiomiocituose (41), o SSN atsakas į tempimą yra įvairių neurotrofinų, įskaitant NAV ir CNTF, kiekio padidėjimas (40). Apibendrinant, dabartiniai tyrimai rodo, kad KEAV daro tiesioginį poveikį nervų sistemai neuronų augimo, išgyvenimo ir neuroapsaugos aspektais, taip pat nustatyta, kad NAV, kuris yra neurotrofinas, yra nauja angiogeninė molekulė, sukelianti įvairų poveikį endotelio ląstelėms. Šį kryžminį KEAV neurotrofinį poveikį ir NAV angiogeninį poveikį išsamiai nagrinėjo Lazarovici ir kt. (72).

6. IŠVADOS

- Stimuliuojant ikislenkstine didelio dažnio elektrine stimuliacija, kardiomiocitai geba sintezuoti angiogeninio veiksnio KEAV kiekius, kas teigiamai veikia ŽVAEL proliferaciją *in vitro*. Tai daug žadantis žingsnis IŠL gydyme. IES sukelta angiogenezė gali būti išeminės širdies ligos gydymo alternatyva.
- IA terapija sumažina pacientų su DKM padidėjusią KEAV koncentraciją. DKMP-IgG sukelia KEAV ekspresiją išaugintuose ŽNKM. Tempimo indukuotą KEAV gamybą ir toliau skatina antikūnai, o dėl DKMP-IgG sukeltos beta1-adrenerginės stimuliacijos padidėjusio susitraukimų dažnio poveikis KEAV indukcijai nenustatytas.
- Kardiomiocitai kontroliuoja savo NAV aukšto dažnio arba nereguliarios stimuliacijos sąlygomis.
- SSN reaguoja į tempimą padidėjusia KEAV ekspresija ir sekrecija. Neuronų KEAV padidėjimas dėl tempimo priklauso nuo laiko ir dozės.

7. TYRIMO APRIBOJIMAI

Nebuvo tirti mechanizmai, kaip IES indukuotas KEAV padidėjimas sukėlė KEAV iRNR sumažėjimą (neigiamas grįžtamasis poveikis). Šiuos mechanizmus galėtų paaiškinti tolimesni tyrimai, kurių metu būtų naudojami KDR receptorių antikūnai IES metu ir analizuojamos tarpląstelinių signalų perdavimo kaskados.

Tyrimai atlikti pasitelkiant žiurkių naujagimių dirbtinai išaugintų kardiomiocitų *in vitro* modelį. KEAV raiška gali reikšmingai skirtis tarp naujagimių ir suaugusių kardiomiocitų. Taip pat neanalizavome IES poveikio esant hipoksijai, kuri stebima esant miokardo infarktui.

Terapinė angiogenezė po miokardo infarkto išlieka daug žadanti, bet sudėtinga širdies ir kraujagyslių medicinos užduotis. Jos svarbą lemia aukštas sergamumas ir mirtingumas ūminių koronarinių sindromų ir jų komplikacijų, tokių kaip aritmija ir širdies nepakankamumas. Kai kurie bandymai indukuoti angiogenezę jau yra ištirti (pvz., su kamieninėmis ląstelėmis, vietiškai skiriant rekombinantinių angiogeninių augimo veiksnių (fibroblastų augimo veiksnių, KEAV).

Šis tyrimas rodo, kad IES gali padidinti endogeninę KEAV raišką skilvelio miocituose, tuo pačiu skatinant endotelio ląstelių proliferaciją, t.y. galimai skatinant angiogenezę. IES gali būti atliekama su stimuliavimo kateteriais, kurie įvedami KS endokardo plote arba per stimuliavimo vielas, įvedamas į vainikinę arteriją (pvz., po revaskuliarizacijos arba net ir į neužsikimšusias smulkiąsias kraujagysles, kurioms negali būti taikoma PKI) arba į venų šakas (vainikinius sinusus). Šis metodas gali būti patrauklus atliekant kitus išeminės širdies ligos *in vivo* tyrimus su gyvūnais modelius, kadangi taikant šį metodą nereikia ląstelių ar baltymų injekcijų ar infuzijų į miokardo taikinius, o stimuliacija viena elektrodų pora apima didelį plotą.

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To my beloved wife - for faith. Brother - for wonderful discussions on scientific topics. Mother and father - for everything. Scientific supervisor for continuous promotion. Colleagues - for the opportunity to go the path of discovery. Good people - for being together.

11. LIST OF PUBLICATIONS

1. Račkauskas G., Saygili E., Rana O.R., Saygili E., Gemein C., Laucevičius A., Aidietis A., Marinskis G., Šerpytis P., Plisienė J., Pauža D.H., Schauerte P. Sub-threshold high frequency electrical field stimulation induces VEGF expression in cardiomyocytes. Cell Transplantation 2015,24(8):1653-1659.
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I

SUB-THRESHOLD HIGH FREQUENCY ELECTRICAL FIELD STIMULATION INDUCES VEGF EXPRESSION IN CARDIOMYOCYTES

Rackauskas G., Saygili E., Rana O.R., Saygili E., Gemein C., Laucevicius A., Aidietis A., Marinskis G., Serpytis P., Plisienė J., Pauza D.H., Schauerte P.

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Subthreshold High-Frequency Electrical Field Stimulation Induces VEGF Expression in Cardiomyocytes

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Subthreshold electrical stimulation (SES) has been shown to induce an improvement of angiogenesis in ischemic and nonischemic skeletal muscles, mediated by increased VEGF expression. VEGF plays a key role in physiological and pathological angiogenesis. Cardiomyocytes possess the ability to synthesize and secrete VEGF. Thus, we thought to investigate the effect of SES on VEGF regulation in cultured neonatal rat ventricular myocytes (NRVMs), in the aim to reveal new techniques for therapeutic angiogenesis in ischemic heart disease. Cell cultures of NRVMs were electrically stimulated with field strengths below the myocyte depolarization threshold (0.5 V/cm with 1 ms bipolar impulse duration). Frequencies ranging from 5 Hz up to 25, 50, and 99 Hz were applied over a period of 48 h. The expression of VEGF and its receptor KDR was determined with Western blot and ELISA. To reveal the biological activity of the secreted VEGF amount, cultured human coronary artery endothelial cells (HCAECs) were treated with the cell culture supernatant of NRVMs exposed to SES. A dominant effect of SES was observed at 25 Hz. Within this particular frequency the VEGF protein amount in the cytoplasm as well as in the cell culture supernatant increased significantly. In parallel, the protein expression of the KDR receptor decreased in a significant manner. Moreover, cell culture supernatant of NRVMs exposed to SES augmented the growth of HCAECs. Cardiomyocytes respond to SES with an increase in biologically active VEGF expression that promotes cell proliferation of HCAECs. This mechanism may provide new approaches to develop therapeutic angiogenesis in the ischemic heart.

Key words: Vascular endothelial growth factor (VEGF); Angiogenesis; Cardiomyocytes; Electrical stimulation

INTRODUCTION

Ischemic heart disease (IHD) due to coronary artery disease (CAD) remains the leading cause of death in the Western world (6). Revascularization of existing arteries and the formation of new collateral vessels may be a promising strategic aim in order to improve the function of organs suffering from hypoxia. Vascular endothelial growth factor (VEGF) plays a pivotal role in angiogenesis and neovascularization. There is accumulating evidence that VEGF is expressed in the heart and that its expression is markedly increased in response to hypoxia or cyclic mechanical stretch (11,13). These (patho)physiological stimuli recent studies have shown that subthreshold electrical stimulation (SES) augments VEGF expression in cultured skeletal muscle cells. Moreover, in vivo SES has been shown to induce vascular angiogenesis in a hindlimb ischemia model in rats mediated by VEGF (4,7). Since SES might be delivered to hypoxic cardiac tissue in vivo (e.g., with catheters or wires) this proof-of-principle study investigated the effect of SES on VEGF expression in cultured neonatal rat ventricular myocytes (NRVMs).

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MATERIALS AND METHODS

Cell Cultures of Neonatal Rat Ventricular Myocytes (NRVMs)

All animal experiments were approved by the local and state Ethics in Animal Research Committee (University Hospital RWTH Aachen, Germany, TV-No. 10596 A). NRVMs were isolated and cultured as described previously by Simpson et al. with little modifications according to the protocol by Zobel et al. (14,16). After cervical dislocation, hearts were obtained from 1- to 3-day old Sprague–Dawley rats with no consideration to the gender (Charles River, Sulzfeld, Germany) and digested with collagenase II (1.0 mg/ml, 280 U/mg, C2-22; Biochrom, Berlin, Germany), trypsin (0.5 mg/ml, trypsin 1:250, L-11-002; PPA, Colbe, Germany), and 1% penicillin–streptomycin (P-4458; Sigma-Aldrich, Steinheim, Germany). Myocytes were purified from fibroblasts by passage through a Percoll gradient (P-1644; Sigma-Aldrich). Myocytes were plated onto six-well dishes (140685; Nunc, Schwerte, Germany) at a density of 3×10^5 cells per well. Cells were grown in DMEM/Ham's F-12 + L-glutamine (FG-4815; Biochrom) supplemented with 10% horse serum (B15-021; PPA) and 5% fetal bovine serum (A-15-101; PPA). After 24 h, the serum medium was removed, and the cells were washed and maintained in serum-free DMEM/Ham's F-12 + L-glutamine.

Human coronary artery endothelial cells (HCAECs) were grown in endothelial cell growth medium (C-22020; PromoCell GmbH, Heidelberg, Germany) until starting the experiments with NRVM cell-conditioned medium. The tissue used by PromoCell for the isolation of human cell cultures is derived from donors who have signed an informed consent form (this being done by the donor himself, an authorized agent, or a legal agent), which outlines in detail the purpose of the donation and the procedure for processing the tissue.

Application of Subthreshold Electrical Simulation (SES)

Three days after cell isolation, serum-containing medium was changed to serum-free medium, and thereafter NRVMs were stimulated for 48 h with low-voltage, high-frequency bipolar electrical field stimulation (HFES) using the C-PaceEP external pacing system (IonOptix, Wageningen, The Netherlands). The stimulus strength (0.5 V/cm, impulse duration 1 ms) was chosen to yield an electrical field that was well below the threshold that elicited contraction of the cultured myocytes. The lowest capture threshold in the different preparations was 1.0 V/cm. Lack of contraction of the myocytes was checked every 12 h during the experiment by microscopic inspection of the cultures. The frequencies chosen were 5 Hz, 25 Hz, 50 Hz, and 99 Hz. Cell cultures without HFES served as controls.

ELISA

For VEGF ELISA, each well was normalized to 300,000 cells, and SES steps from 5 Hz up to 25, 50, and 99 Hz were analyzed. The conditioned media was collected at 0, 24, and 48 h after exposure to SES, and protein in the cell culture supernatant was concentrated by using the vivaspin columns from Sartorius (Göttingen, Germany). Samples were assayed by a VEGF ELISA kit from R&D Systems (Minneapolis, MN, USA) with normalized protein amounts according to the manufacturer's instructions. Protein amount was quantified by using the NanoDrop 1000 from Thermo Scientific (Wilmington, DE, USA).

Western Blot

Western blots (WB) to assess VEGF and KDR proteins were performed with normalized protein amounts. For WB, cells were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0; #9090.2; Carl-Roth, Karlsruhe, Germany), 1% nonident P-40 (#74.385; Merck, Darmstadt, Germany), and 10% glycerol (#50405-1; Biomol, Hamburg, Germany). Cell lysates were cleared by centrifugation at $17{,}000 \times g$ for 20 min. Extracts (100 µg) were subjected to electrophoretic separation through a 10% SDS-polyacrylamide gel (SDS #51430-2, Biomol/polyacrylamide gel #3029.1; Carl-Roth) and subsequently transferred to a polyvinylidene difluoride membrane (#RPN303F; GE-Healthcare Life Sciences, Freiburg, Germany). The membrane was blocked overnight with 5% bovine serum albumin (#01400-1; Biomol) in phosphate-buffered saline (#L-1825; Biochrom) containing 0.1% Tween 20 (PBST; #TW0020; Rockland, ME, USA). Primary rabbit anti-GAPDH (1:1,000; #2118; Cell Signaling, Danvers, MA, USA), rabbit anti-VEGF (1:150; 19003-1-AP; Acris Antibodies, Herford, Germany), and rabbit anti-KDR (200 µg/ml, 1:100; sc-315; Santa Cruz, Heidelberg, Germany) were incubated overnight. Blots were washed three times with PBST and incubated with goat anti-rabbit horseradish peroxidase-conjugated antibody (GE Healthcare) for 1 h. Finally, the ECL Advance Western Blotting Detection Kit (GE Healthcare Life Sciences) was used to visualize the bands with the advanced luminescent image analyzer LAS-3000 from Fujifilm (Tokyo, Japan). Relative densitometry analyses of the bands were performed with the Multi-Gauche V3.0 software from Fujifilm.

Antibodies

Cells were fixed in 4% paraformaldehyde (8.187.15; KMF, Lohmar, Germany), permeabilized with Triton X-100 (50800-1; Biomol), and blocked with 1% BSA (01400-1; Biomol). Cells were incubated with the primary antibody, (1:150) rabbit anti-VEGF (200 µg/ml,

1:150; sc-507; Santa Cruz) mouse anti-Trop T-C (200 µg/ml; sc-20025; Santa Cruz) overnight at 4°C and then with a goat anti-rabbit (1:100) and goat anti-mouse (1:100) secondary antibody for 2 h coupled to Alexa Fluor 488 (VEGF) (1 mg/ml; A11008; Invitrogen) or Alexa Fluor 647 (Trop T-C) (1 mg/ml; A20990; Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (10236276001; Roche Mannheim, Germany). To visualize fluorescence signals, the Axiovert 200M microscope and the AxioVision Rel. 4.5 software from Zeiss (Jena, Germany) were used.

Primary antibodies for Western blot: rabbit anti-GAPDH (1:1,000; #2118; Cell Signaling), rabbit anti-VEGF (1:150; 19003-1-AP; Acris Antibodies), rabbit anti-KDR (200 µg/ml, 1:100; sc-315; Santa Cruz).

RNA Preparation, First-Strand cDNA Synthesis, and Quantitative Real-Time Reverse Transcription-PCR

Total RNA was extracted from NRVMs using the Qiagen RNeasy Mini Kit (Venio, The Netherlands) and following the manufacturer's instructions. A total of 1 µg of RNA were reverse-transcribed using random hexamers from the Fermentas First strand cDNA Synthesis Kit (#K1622; Fermentas GmbH, St. Leon-Rot, Germany).

Quantitative Real-Time Reverse-Transcription-PCR

Real-time PCR was performed in 96-well plates on the Step-One plus Sequence Detection System (ABI) as described previously (9). Data were collected with instrument spectral compensation by Applied Biosystems SDS 1.2.3 software (Applied Biosystems, Foster City, CA, USA).

Primers and Probes for Quantitative Real-Time Reverse-Transcription-PCR

PCR primers and fluorogenic probes for the target gene and the endogenous control were purchased as Assays-On-Demand (Applied Biosystems). The assay numbers for the endogenous control and target genes were as follows: Rn00560865_m1 (β-2 microglobulin), Rn01533872_m1 (Ngfb), Rn00561661_m1 (Nppa). The assay numbers were as follows: Rn01511602_m1 (VEGF), Rn00564986_m1 (KDR), Rn00560865_m1 (β-2 microglobulin),

Investigation of Human Coronary Artery Endothelial Cell (HCAEC) Proliferation

To reveal the proliferation of HCAECs due to an effect of biologically active VEGF secreted by NRVMs exposed to SES, we used a colorimetric Assay kit from PromoCell (WST-8). WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product that is soluble in cell culture medium. The amount of formazan is directly proportional to the number of living cells. Therefore, this method allows sensitive determination of the number of viable cells in cell culture.

HCAECs were incubated for 48 h with NRVM-conditioned medium. The frequencies of 25 Hz and 5 Hz were analyzed, due to the fact that the dominant effect of VEGF induction was observed at 25 Hz (compared to 5 Hz and control).

Statistical Analysis

All values are expressed as mean ± SEM. Data were processed using Statistical Package for Social Sciences release 14.0 for Windows (IBM, Somers, NY, USA). The statistical significance of differences was evaluated by the Student's *t*-test for two groups and one-way ANOVA followed by LSD post hoc test for multiple groups. A value of $p < 0.05$ was accepted as statistically significant.

RESULTS

SES Augments VEGF Expression and Secretion in Neonatal Rat Ventricular Myocytes (NRVMs)

To analyze the effect of SES on cardiomyocyte VEGF expression, NRVMs in cell culture were stimulated for 48 h at 5 Hz, 25 Hz, 50 Hz, and 99 Hz. VEGF protein amount was measured from cell culture medium at 24 and 48 h of stimulation. HFES elicited an increase in VEGF expression both in the cytoplasm and supernatant. A dominant effect of SES was observed at 25 Hz with a 1.8-fold increase of VEGF protein amount in the cytoplasm (Fig. 1). In parallel, VEGF in the cell culture supernatant

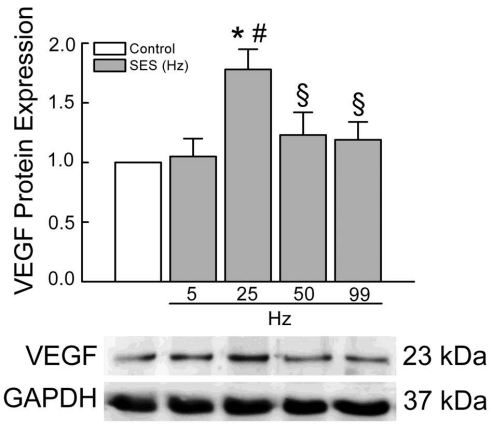

Figure 1. Frequency dependence of relative VEGF protein expression in cell lysates during 48 h of subthreshold electrical stimulation (SES). Results are representative of at least five independent experiments. *$p < 0.05$ versus control. #$p < 0.05$ versus 5 Hz. §$p < 0.05$ versus 25 Hz.

rose significantly with an almost bell-shaped dose–response curve and a maximum at 25 Hz. The effect of HFES on VEGF expression was sustained for at least 48 h at all HFES frequencies (Fig. 2). Figure 3 shows VEGF immunocytofluorescence images of the VEGF expression in cardiomyocytes, which were exposed to subthreshold high-frequency electrical fields in vitro.

In order to evaluate which was the relative contribution of augmented VEGF gene expression on elevated VEGF protein expression, we analyzed the VEGF mRNA content of cell lysates.

Changes of VEGF mRNA and VEGF Receptor During HFES in NRVMs

HFES led to significant decreases in VEGF mRNA, which was significant for 25, 50, and 99 Hz with a maximum at 25 Hz (Fig. 4). Since this might be due to a negative feedback loop in response to the increased VEGF protein levels both in the cytoplasm and in the supernatant, we measured the expression of the membranous VEGF receptor (KDR) both on protein (Fig. 5) and mRNA levels (Fig. 6). SES significantly decreased the expression of the KDR receptor protein at 25 Hz and—though not significantly—at 50 and 99 Hz. Likewise, the KDR receptor mRNA expression was significantly augmented during SES at 25, 50, and 99 Hz (Fig. 6). Of note, the relative suppression of VEGF mRNA expression and KDR protein and gene expression followed the frequency dependency as observed for the induction of VEGF protein with a maximum at 25 Hz.

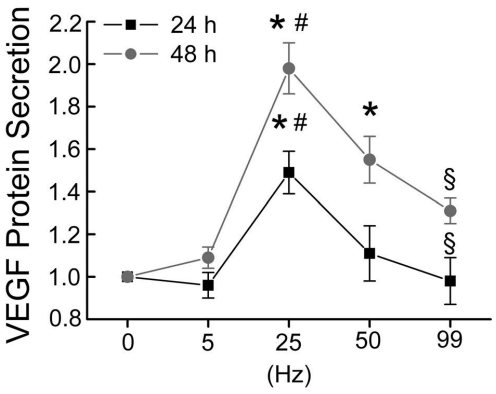

Figure 2. Extracellular secretion of VEGF protein normalized to control. Medium was collected at 24 and 48 h during continuous electrical stimulation, and VEGF protein was determined by ELISA. Results are representative of at least five independent experiments. *$p<0.05$ versus control. #$p<0.05$ versus 5 Hz. §$p<0.05$ versus 25 Hz.

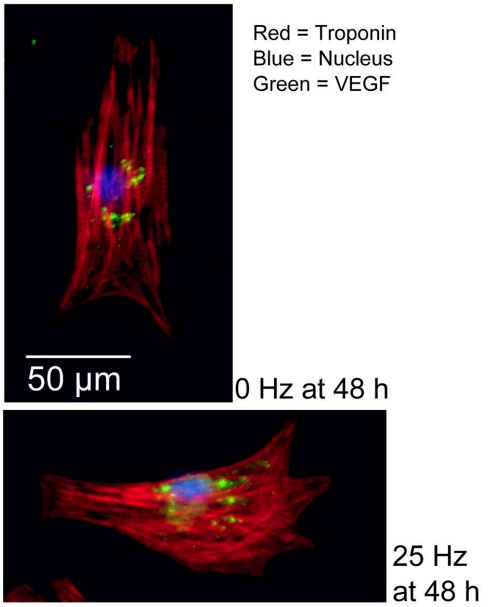

Figure 3. Representative images of NRVMs in cell culture exposed to SES. These images show abundance of VEGF protein.

SES-Induced VEGF Expression Is Biologically Active and Promotes Human Coronary Artery Endothelial Cell Proliferation

To investigate whether the observed VEGF induction by SES is effective in promoting endothelial cell proliferation, HCAECs were incubated with NRVM-conditioned medium (48 h of SES at 5 Hz, 2 5Hz, 50 Hz, and 99 Hz, respectively). We found that medium conditioned by 25 Hz SES augmented the growth of HCAECs, whereas the medium conditioned by 5 Hz SES did not elicit a significant growth response of HCAECs in vitro (Fig. 7).

DISCUSSION

The principal findings of our study are (a) SES of cultured NRVMs elicits a significant increase in VEGF protein expression, (b) the dominant frequency of VEGF induction by SES was 25 Hz, (c) NRVM VEGF secretion due to SES is biologically active and promotes HCAEC proliferation in vitro.

The most dominant effect of SES on VEGF expression was present at 25 Hz with an almost twofold increase in extracellular VEGF protein levels. A frequency dependency of VEGF expression during in vitro SES was previously described in other cell lines with the optimal electrical frequency varying for different cell types, that is, 50 Hz for

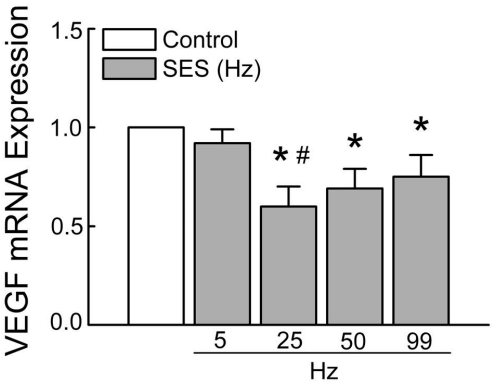

Figure 4. Relative expression of VEGF mRNA in cultured NRVMs. Cells were electrically stimulated for 48 h at indicated frequencies. Thereafter, total cDNA was extracted, and RT-PCR for VEGF mRNA was performed. Results are representative of at least four independent experiments. *$p < 0.05$ versus control. #$p < 0.05$ versus 5 Hz.

skeletal muscle cells and 24 Hz for smooth muscle cells (5). The exact mechanism by which HFES augments cellular protein expression is unclear. In myocardial or cardiac neuronal cells, the functional excitatory effects of electrical stimulation (e.g., muscle cell contraction or neuronal firing) depends on the membrane depolarization, which in turn is determined by its refractory period, with higher frequencies being typically effective in cardiac neuronal cells due to its shorter refractory periods. Trophic effects of SES have also been described in sympathetic or parasympathetic cardiac neuronal cells (reference our group). High-frequency stimulation led to an increased NGF and NT-3 protein and gene expression with subsequent induction of neuronal cell growth and hypertrophy in vitro and in vivo (8). However, as opposed to the present study, this effect was dependent on neuronal cell membrane depolarization, since suprathreshold current was delivered, and the effect was blunted by lidocaine (10).

In this study, subthreshold electrical stimuli that were not capable of myocardial depolarization. Thus, different electrical effects than only cell membrane depolarization may be operative.

Most notably, SES seems to represent a ubiquitous mechanism by which cellular expression of growth factors can be enhanced. This is further supported by the observation of a frequency-dependent increase in insulin-like growth factor-2 protein and gene expression in osteosarcoma cells (1). In fact, this effect may not solely be restricted to the induction of growth factors, since an increase in constitutive NO synthase during SES of cardiomyocytes has also been described (5).

Theoretically, SES could increase the stability of VEGF protein. However, Kanno et al. showed that electrical stimulation did not affect the stability of VEGF mRNA. This is evidence that the augmentation of VEGF mRNA by electrical stimulation occurs predominantly at the transcriptional level. In addition, transient electrical stimulation (2 h vs. 24 h) led to almost identical VEGF mRNA levels indicating that the initial mRNA increase elicited by SES was sufficient to induce a sustained augmentation of VEGF protein synthesis. This is further supported by the observation of a later onset of the increase in VEGF protein expression 12 h after initiation of SES with a subsequent maximum at 48 h (4).

In the present study, the VEGF mRNA amount was decreased after 48 h of SES despite an increase in VEGF protein. Since KDR receptor mRNA and protein were decreased, a negative feedback mechanism of increased VEGF protein levels on VEGF mRNA expression via downregulated KDR expression might be operative. In fact, Hang and colleagues have already shown that the VEGF mRNA expression gradually decreases during prolonged electrical stimulation and that VEGF-induced angiogenesis may be under a negative feedback control (3). Thus, if a limited exposure of SES is capable of inducing an increase in VEGF mRNA sufficient to induce a sustained VEGF protein expression, the reflectory downregulation of VEGF mRNA expression may already occur within the 48-h stimulation period, thus yielding overall lower mRNA levels than in the control group.

Of note, a negative feedback loop secondary to SES-induced VEGF upregulation might counteract the beneficial effect of SES on therapeutic angiogenesis. Since, in a potential clinical application scenario, SES may only

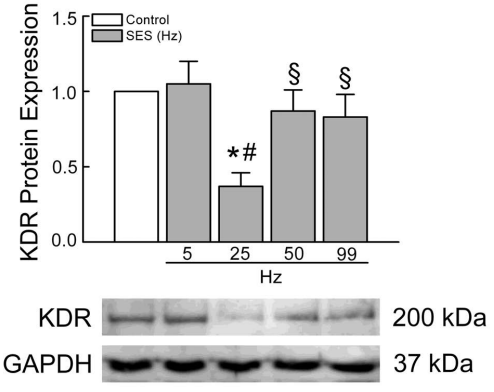

Figure 5. Relative KDR protein expression. Results are representative of at least five independent experiments. *$p < 550.05$ versus control. #$p < 50.05$ versus 5 Hz. §$p < 50.05$ versus 25 Hz.

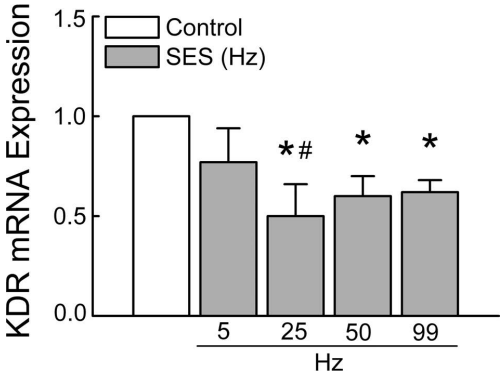

Figure 6. Relative expression of KDR mRNA in cultured NRVMs. Cells were electrically stimulated for 48 h at indicated frequencies. Results are representative of at least four independent experiments. *$p<0.05$ versus control. #$p<0.05$ versus 5 Hz.

be applied on a short-term basis (e.g., via an intracardiac wire or catheter), the induction of angiogenesis may still be sufficient for a clinically meaningful effect on revascularization. Such a hypothesis is supported by the present study in which a short-term SES exposure of 48 h led to a VEGF protein increase sufficient to induce a cellular growth in vitro.

There are several compelling studies showing the beneficial effects of chronically applied electrical field stimulation to various cell culture and animal models. A study by Shafy and colleagues demonstrated that chronic electrical stimulation via a cardiac resynchronization therapy device contributes to cell differentiation of autologous cultured myoblasts, injected into the infracted areas in a sheep model of myocardial infarction. The authors could show that this technical approach resulted in an improvement in myocardial function (12). Recently Haneef et al. furthermore demonstrated that electrostimulation of stem cells is a safe and effective approach for cell survival and differentiation into cardiac cells (2). Regarding the role of VEGF, Spadaccio and coworkers revealed in an in vivo model of myocardial infarction that continued electrical stimulation contributes to an improvement in myocardial function paralleled by an increase in angiogenesis through endothelial progenitor cell migration and VEGF production (15). These novel studies encourage the investigation of myocardial tissue engineering to develop new therapeutic tools for ischemic heart disease. Our results are completely in line with these findings. Moreover, in the field of new therapeutic approaches for the treatment of heart diseases, it is likely safer to have the opportunity to perform SES to avoid side effects like arrhythmias.

Study Limitations

We did not investigate the mechanisms by which SES-induced VEGF augmentation affected the decrease in VEGF mRNA (negative feedback loop). Future experiments using KDR receptor antibodies during SES and analysis of the intracellular signaling cascades leading to the downregulation of VEGF mRNA may further elucidate these mechanisms.

In the present study, we used an in vitro model of cultured CM from neonatal rats. The expression pattern of VEGF may vary significantly between neonatal and adult rat cardiomyocytes. In addition, we did not analyze the SES response in hypoxic myocytes, as they may be found in the infarction border zone.

Therapeutical angiogenesis after myocardial infarction remains a promising, but challenging, task for cardiovascular medicine. Its importance stems from the high morbidity and mortality due to sequels of occlusive CAD like arrhythmias and heart failure. Several attempts to induce angiogenesis have been investigated (like stem cells, local administration of recombinant angiogenetic growth factors: fibroblast growth factor, VEGF).

The current proof-of-concept study shows that SES is capable of boosting endogenous VEGF expression in ventricular myocytes, which in turn is able to induce proliferation of endothelial cells, thus potentially inducing angiogenesis. SES may be delivered via stimulating catheters positioned either at the endocardial site of the LV or via stimulating (PCI) wires, which may be introduced into the coronary artery (e.g., after revascularization or even in nonoccluded small vessels not amenable to PCI) or accompanying venous vessels (coronary sinus tributaries). The fact that this approach does not rely on the

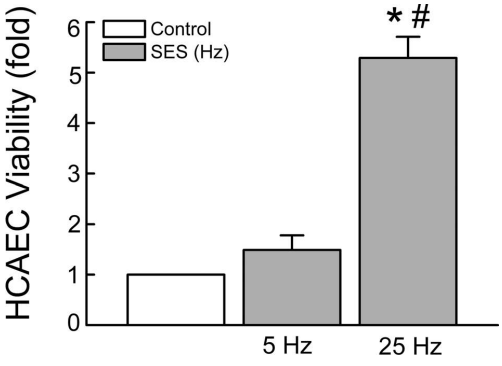

Figure 7. Growth response of HCAECs incubated with cell-conditioned medium of NRVMs exposed to SES. Conditioned culture supernatant, 25 Hz, was sufficient to promote endothelial cell proliferation. Results are representative of at least four independent experiments. *$p<0.05$ versus control. #$p<0.05$ versus 5 Hz.

introduction of cells or proteins in myocardial targets by injection or infusion and stimulation from a single electrode pair may cover a larger area makes this approach potentially attractive for further in vivo studies in animal models of CAD.

CONCLUSIONS

Cardiomyocytes stimulated by low-voltage electrical fields produce potent amounts of the angiogenetic factor VEGF, which has positive proliferation effects on HCAECs in vitro. This is a very potent step forward to the treatment of IHD. Angiogenesis due to SES may be one optional alternative treatment of CAD.

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II

AUTOANTIBODIES IN DILATED CARDIOMYOPATHY INDUCE VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION IN CARDIOMYOCYTES.

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Autoantibodies in dilated cardiomyopathy induce vascular endothelial growth factor expression in cardiomyocytes

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ABSTRACT

Background: Autoantibodies have been identified as major predisposing factors for dilated cardiomyopathy (DCM). Patients with DCM show elevated serum levels of vascular endothelial growth factor (VEGF) whose source is unknown. Besides its well-investigated effects on angiogenesis, evidence is present that VEGF signaling is additionally involved in fibroblast proliferation and cardiomyocyte hypertrophy, hence in cardiac remodeling. Whether autoimmune effects in DCM impact cardiac VEGF signaling needs to be elucidated.

Methods: Five DCM patients were treated by the immunoadsorption (IA) therapy on five consecutive days. The eluents from the IA columns were collected and prepared for cell culture. Cardiomyocytes from neonatal rats (NRCM) were incubated with increasing DCM-immunoglobulin-G (IgG) concentrations for 48 h. Polyclonal IgG (Venimmun N), which was used to restore IgG plasma levels in DCM patients after the IA therapy was additionally used for control cell culture purposes.

Results: Elevated serum levels of VEGF decreased significantly after IA (Serum VEGF (ng/ml); DCM pre-IA: 45 ± 9.1 vs. DCM post–IA: 29 ± 6.7; $P < 0.05$). In cell culture, pretreatment of NRCM by DCM-IgG induced VEGF expression in a time and dose dependent manner. Biologically active VEGF that was secreted by NRCM significantly increased BNP mRNA levels in control cardiomyocytes and induced cell-proliferation of cultured cardiac fibroblast (Fibroblast proliferation; NRCM medium/HC-IgG: 1 ± 0.0 vs. NRCM medium/DCM-IgG 100 ng/ml: 5.6 ± 0.9; $P < 0.05$).

Conclusion: The present study extends the knowledge about the possible link between autoimmune signaling in DCM and VEGF induction. Whether this observation plays a considerable role in cardiac remodeling during DCM development needs to be further elucidated.

1. Introduction

Circulating autoantibodies that target diverse myocardial structures have been identified as major predisposing factors for dilated cardiomyopathy (DCM) [1]. Patients with DCM show elevated serum levels of vascular endothelial growth factor (VEGF) whose source is unknown [8]. Even though a large number of studies suggest that VEGF is beneficial in ischemic cardiomyopathies due to its well-investigated effects on angiogenesis and neovascularisation [2–4], to the best knowledge of the authors there is no data available reporting a beneficial role of VEGF in DCM. In contrast, recent data indicate that an un-physiological expression and secretion of VEGF might be harmful via its pleiotrophic effects on all cell types involved in cardiac remodeling. Accumulating data suspect VEGF as a cardiac transcriptional regulator, which is crucially involved in physiological and pathological hypertrophy signaling in cardiomyocytes [5]. In this context, Zhou et al. could convincingly demonstrate that VEGF signaling plays a pivotal role

Abbreviations: DCM, Dilated cardiomyopathy; ICM, Ischemic cardiomyopathy; IA, Immunoadsorption; IgG, Immunoglobulin G; LVEF, Left ventricular ejection fraction; VEGF, Vascular endothelial growth factor; BNP, brain natriuretic peptide; NRCM, Neonatal rat cardiomyocytes; NYHA, New York Heart Association.

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in phenylephrine induced cardiomyocyte hypertrophy [6]. Additionally, Tsoporis and colleagues have recently shown that VEGF, which is released by cardiomyocytes promotes proliferation of cardiac fibroblasts [7]. It is still unclear whether these different observations can be predictably translated into cardiac remodeling. Nevertheless, since VEGF levels are increased in DCM patients, the question raises whether VEGF is just compensatory elevated or it is directly involved in cardiac remodeling pathways due to unknown mechanisms [8–10].

The current options to inhibit or to reverse the process of structural remodeling in the failing heart are limited. Drugs targeting the beta1-adrenergic or the angiotensin signaling pathways can improve survival but have a narrow therapeutic range due to side-effects including bradycardia and/or hypotension [11,12]. There is accumulating evidence that the therapy of immunoadsorption (IA) leads to a long-term improvement of cardiac function in patients suffering from DCM [13–16]. Whether autoantibodies in DCM influence cardiac VEGF expression, and whether an un-physiological VEGF secretion might promote remodeling mechanisms due to hypertrophy signaling and cardiac fibroblast proliferation is rather unexplored.

Thus, we hypothesized that autoantibodies in DCM induce the expression of VEGF in cardiomyocytes that might contribute to a hypertrophy response and cardiac fibroblast proliferation. Furthermore, since DCM is accompanied by an increase in heart rate and mechanical overload, we additionally analyzed the impact of both factors on the expression of VEGF by cardiomyocytes.

2. Methods

2.1. Study design, patient characteristics and IgG collection by immunoadsorption (IA)

Approval for the study (No. EK 095/09) was obtained from the local ethics committee at RWTH Aachen University Hospital in accordance with International guidelines for Good Clinical Practice. The investigation also conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985). DCM patients ($n = 5$, mean age 50.3 ± 3.5 years) with severe left ventricular dysfunction (LVEF < 30%, New York Heart Association class III) were treated with IA on five consecutive days. After the final IA session, all patients received 0.5 g/kg polyclonal IgG (Venimmun N) to restore IgG plasma levels [17]. The same polyclonal IgG pool was used for cell culture control purposes in a matched concentration, and is indicated as healthy control IgG (HC-IgG) in the appropriate graphs. All DCM patients had a history of heart failure of at least 1 year and had a stable dosage of oral medications including angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, diuretics, aldosterone antagonists and β-blockers for at least 6 months. Ischemic disease was excluded by angiography. The IA therapy was performed with protein A Immunoadsorba columns (Fresenius Medical Care AG, Bad Hombug, Germany). Column eluent (CE) from IA columns containing the eliminated IgG was collected and prepared for cell culture experiments as described elsewhere [16]. The Nanodrop ND-1000 (PeqLab, Erlangen, Germany) was used to determine the protein concentration of the CE. Aliquots of CE were stored at −80 °C until use. Serum IgG levels of our DCM patients were monitored and an average reduction in IgG levels of 91.7% could be observed, as measured at the end of the last session on the fifth day of IA. Ischemic cardiomyopathy (ICM) patients ($n = 7$) showed similar parameters regarding ejection fraction, LV-dimensions and the NYHA classifications as compared to DCM patients (data not shown).

2.2. Cell culture

All animal experiments were approved by the local Ethics in Animal Research Committee (University Hospital RWTH Aachen, Germany, TV-No.: 10596 A). Neonatal rat ventricular myocytes (NRCM) from Sprague Dawley rats (Charles River, Erkrath, Germany) were isolated and cultured at a density of 1×10^5 cells/well on 6 well plates and grown for 2 days before starting the experiments [18]. Cardiomyocytes were purified from cardiac fibroblasts through gradient centrifugation. Cardiac fibroblasts were cultured at a density of 1×10^5 cells/well. "n" represents the number of cell preparations. For each cell preparation we used an average of 15–20 neonatal rats. To determine the contraction rate of cultured NRCM, the dishes were transferred to the stage of an inverted microscope (Nikon). 15 circular fields of each well were examined through the perforations of a template. The data represent observations on 50 cells or cell clusters of synchronously contracting myocytes from five different cell preparations. Human coronary artery endothelial cells (HCAEC) were purchased from Promocell (Heidelberg, Germany) and seeded at a density of 1×10^5 cells/well. To determine the amount of cell proliferation the colorimetric cell viability Kit II (WST-1) from Promokine (Heidelberg, Germany) was used according to the manufacturer's instructions.

2.3. Mechanical stretch

NRCM were incubated on collagen-coated silicone membranes and static stretch was introduced to attached myocytes by applying a gradual increase of tension as described previously [19]. Stretch was increased stepwise every 24 h from 3% to 13% (steps: 3, 7, 13%).

2.4. ELISA and Western blot

For VEGF ELISA each well was normalized to 1×10^5 cells/well. The conditioned media was collected at 0–24 and 48 h after starting the experiments. Samples were assayed by a VEGF ELISA kit from R&D Systems (Minneapolis, USA) according to the manufacturer's instructions. The same assay was used to determine serum levels of VEGF from patients with DCM and ischemic cardiomyopathy (ICM). Western blots were performed with normalized protein amounts of 100 μg/slot as described previously [20].

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

RNA extraction, first-strand cDNA synthesis and quantitative real-time PCR experiments were performed as described previously [18]. PCR primers and fluorogenic probes for VEGF, BNP and the endogenous control were purchased from Applied Biosystems (Foster City, CA). The assay numbers were as follows: Rn01511602_m1 (VEGF), Rn00580641_m1 (BNP), Rn00560865_m1 (β-2 microglobulin).

2.6. TUNEL assay

The TUNEL assay was performed using the APO-BrdU TUNEL Assay Kit (Invitrogen), according to the manufacturer's instructions. Cell apoptosis was determined by counting the number of TUNEL-positive nuclei and expressed as percentage of total cells counted [18].

2.7. Antibodies

Primary antibodies for Western blot: rabbit anti-GAPDH (Cell Signaling, #2118), goat anti-$VEGF_{164}$ (R&D Systems, #AF564), rabbit

anti-VEGF neutralizing antibody (abcam, #ab9570), goat anti-VEGF receptor 2 (abcam, #ab10972).

2.8. Statistical analysis

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

3. Results

3.1. Augmented serum levels of VEGF in patients with DCM decrease after IA

Since increased myocardial wall stress can affect VEGF expression, the VEGF serum levels of DCM patients were compared to that of patients with ischemic cardiomyopathy (ICM). The left ventricular ejection fraction as well as left ventricular end-systolic and end-diastolic diameters of the two groups showed no significant differences (data not shown). In our small number of patients we observed significantly higher baseline VEGF serum levels in the group of DCM as compared to the patients in the group of ICM. Four weeks after the IA therapy we observed a sizeable decrease of serum VEGF levels in the group of DCM patients compared to the baseline levels (Fig. 1A). At this point, the serum VEGF levels in both groups (DCM vs. ICM) showed nearly the same VEGF concentrations, without any significant differences in the above-mentioned echocardiographic parameters.

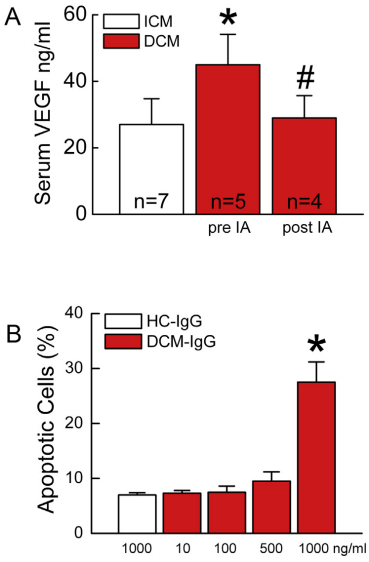

Fig. 1. A) Serum levels of VEGF decrease after the IA therapy. VEGF serum levels in patients with DCM showed significantly higher baseline values as compared to ICM patients. After the IA therapy, serum VEGF levels showed almost the same concentration in both groups. In our small number of patients, this decrease was not paralleled by statistically significant alterations in echocardiographic parameters (data not shown). The number of patients post IA decreased to n = 4, since one of the 5 DCM patients who initially participated the study received a heart transplantation during the study period. *p < 0.05 vs. ICM, #p < 0.05 vs. DCM. B) Dose dependent effect of DCM-IgG on the rate of apoptosis in NRCM.NRCM were incubated with healthy control (HC) IgG and DCM-IgG. Apoptosis was determined by TUNEL assay. At a dosage of 500 ng/ml DCM-IgG a slight increase in apoptosis could be observed, which became significant at a concentration of 1000 ng/ml. Pretreatment of NRCM with HC-IgG remained without effect on apoptosis. HC-IgG = polyclonal IgG (Venimmun N) to restore IgG plasma levels, DCM-IgG = IgG from patients with DCM. *p < 0.05 vs. HC-IgG and DCM-IgG 10, 100, 500 ng/ml. All n = 5 cell preparations.

3.2. Pre-treatment of NRCM by DCM-IgG induce the expression of VEGF

In cell culture, we first investigated the dose response of DCM-IgG on NRCM with respect to apoptosis. We observed that the

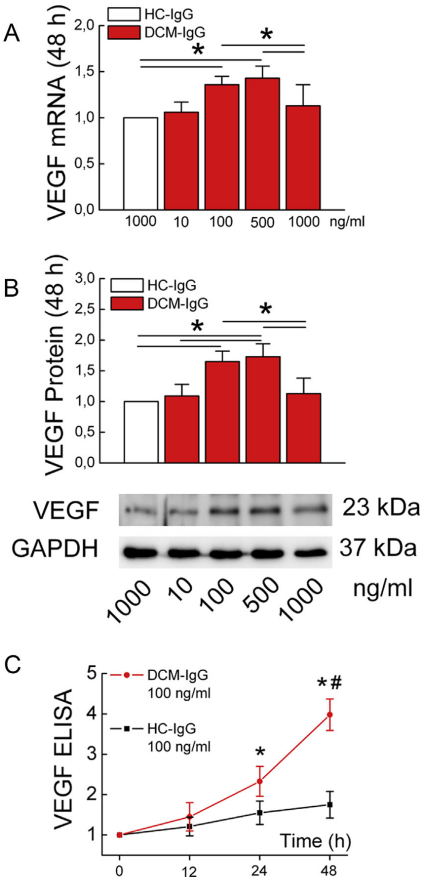

Fig. 2. The expression of VEGF is increased in NRCM due to DCM-IgG stimulation. Treatment of NRCM with DCM-IgG at a concentration of 100 ng/ml lead to an increase in VEGF expression both on mRNA and protein levels as compared to the group of NRCM treated with healthy control IgG at a concentration of 1000 ng/ml. At a DCM-IgG concentration of 1000 ng/ml VEGF levels decreased probably as a result of cell apoptosis (A, B). The time dependent increase in VEGF secretion due to DCM-IgG stimulation was determined by ELISA experiments (C). A significant increase in VEGF secretion could be observed after 24 h of 100 ng/ml DCM-IgG stimulation as compared to 0 h. After 48 h of 100 ng/ml DCM-IgG stimulation the increase in VEGF expression became also significant if compared to the control cell group, which was stimulated by HC-IgG at the same concentration of 100 ng/ml. A, B: *p < 0.05, C: *p < 0.05 vs. 0 h, #p < 0.05 vs. control cells. All n = 5 cell preparations.

degree of apoptosis increased slightly beyond baseline levels at a DCM-IgG concentration of 500 ng/ml (Fig. 1B). Thus, to avoid side effects due to cell death all further experiments were carried out with DCM-IgG concentrations not above a dosage of 100 ng/ml. At this concentration, NRCM did not show an increase in apoptosis, but a significant increase in VEGF expression, both on mRNA and protein levels (Fig. 2A, B). At a DCM-IgG concentration beyond 500 ng/ml this effect was reversed, with VEGF levels receding to the range of control cells, possibly due to an increase in cell death. Using the ELISA technique the secretion of VEGF in the cell culture supernatant could be investigated in a time dependent manner. At a DCM-IgG concentration of 100 ng/ml, the increase in VEGF secretion became statistically significant after 24 h as compared to 0 h, and after 48 h this difference was significant as compared to the control cells treated by HC-IgG (Fig. 2C). NRCM treated with 100 ng/ml of IgG from healthy donors did not show any relevant alteration of VEGF secretion during a period of 48 h.

3.3. Mechanical stretch, but not contraction rate increases VEGF expression in NRCM

DCM is accompanied by an increase in heart rate and mechanical overload. Therefore we analyzed the effects of both parameters on the expression of VEGF on a cellular basis. At a DCM-IgG concentration of 100 ng/ml a significant increase in contraction rate of NRCM could be observed by microscopic examination (Fig. 3A). Higher DCM-IgG concentrations resulted in a decrease in contraction rate, probably due to cell damage ultimately leading to apoptosis, whereas control cells that were incubated with equal IgG concentrations from healthy donors remained unaffected. Pretreatment of NRCM with metoprolol, a selective beta1-adrenergic receptor blocker, resulted in a decrease in contraction rate without any influence on VEGF expression (Fig. 3B, C). By contrast, mechanical stretch lead to an increase in NRCM VEGF expression (Fig. 3D). Co-incubation of stretched cells with DCM-IgG at a concentration of 100 ng/ml showed an additive effect on VEGF induction (VEGF protein expression; control: 1 ± 0.0 vs. stretch 13%: 1.75 ± 0.15 ($P < 0.05$) vs. stretch 13% + 100 ng/ml DCM-IgG: 1.95 ± 0.23).

3.4. VEGF, which is released by NRCM due to DCM-IgG treatment promotes hypertrophy of cardiomyocytes and proliferation of cardiac fibroblasts

Control cardiomyocytes in cell culture responded to the supernatant of DCM-IgG treated cardiomyocytes with an increase in BNP mRNA expression (Fig. 4A). Antibody based specific neutralization of VEGF signaling reversed the observed increase in BNP mRNA as a marker for cardiomyocyte hypertrophy (Fig. 4A). The biological activity of VEGF that was secreted by NRCM due to DCM-IgG stimulation was further confirmed by experiments containing HCAEC and cardiac fibroblasts. Both cell lines responded to the exposure of equal volumes of preconditioned NRCM cell culture supernatant with an increase in cell proliferation (Fig. 4C, B). Antibody-based specific inhibition of VEGF signaling either by receptor blockade or by VEGF neutralization confirmed that the observed increase in cell proliferation was partly induced by biologically active VEGF that was secreted by NRCM due to DCM-IgG stimulation (Fig. 4C, B), since the increase in cell proliferation was significantly inhibited, but not completely blunted.

4. Discussion

4.1. The present study shows

a) The IA therapy decreases elevated VEGF serum levels in patients with DCM. b) DCM-IgG induce VEGF expression in cultured NRCM. c) Stretch-induced VEGF production is further enhanced by such autoantibodies, whereas the increase in contraction rate due to DCM-IgG induced beta1-adrenergic stimulation does not seem to play a role in VEGF induction. d) VEGF that is released by cardiomyocytes due to DCM-IgG stimulation is biologically active and contributes to hypertrophy of cardiomyocytes and proliferation of cardiac fibroblasts.

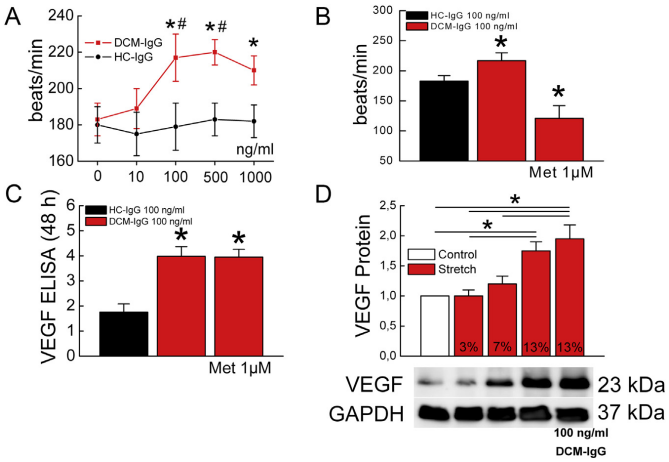

Fig. 3. Mechanical stretch, but not contraction rate contributes to the increase in VEGF expression in NRCM. The contraction rate of cultured cardiac myocytes increased dose dependently with a maximum at 100 ng/ml of DCM-IgG stimulation (A). Blockade of the beta-1 adrenergic signaling by metoprolol decreased the contraction rate without any effect on VEGF expression (B, C). Mechanical stretch-induced VEGF induction was enhanced by DCM-IgG stimulation (D, right panel 13% stretch with 100 ng/ml DCM-IgG). Met = Metoprolol. A: *p < 0.05 vs. 0 ng/ml of DCM-IgG, #p < 0.05 vs. HC-IgG. B, C: *p < 0.05 vs. HC-IgG, #p < 0.05 vs. DCM-IgG. D: *p < 0.05. All n = 5 cell preparations.

4.2. The role of autoantibodies in VEGF induction

Autoimmune mediated angiogenic signaling has been described e.g. in systemic lupus erythematosus or myasthenia gravis (MG) [21,22]. In the latter, the authors could demonstrate a relevant positive correlation of VEGF levels in patients with MG as compared to healthy controls. Furthermore, in cancer research pro- and anti-angiogenic factors are known to be crucially involved in tumor progression and disease development [23,24]. VEGF is the main potent angiogenic factor responsible for the formation of new vessels. Up to now, the induction of VEGF in cancer cells is not fully understood. Recently, Lombardi and colleagues could convincingly demonstrate that breast cancer IgG increased the expression of VEGF in tumor cells. This response was promoted via muscarinic receptor signaling [25]. These findings and our present results clearly demonstrate that autoantibodies in various diseases and pathological circumstances are potent inducers of growth factors, especially VEGF.

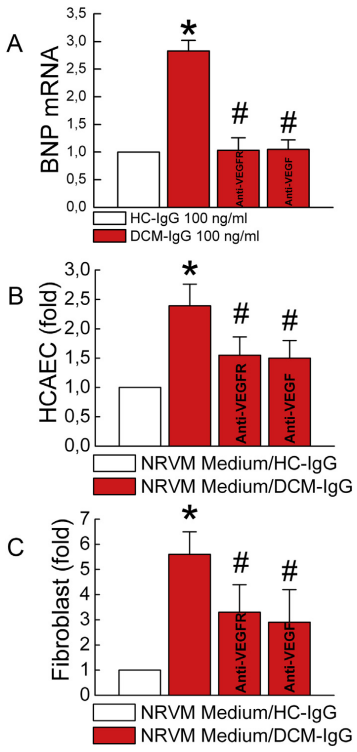

Fig. 4. A) DCM-IgG stimulation promotes cardiomyocyte hypertrophy. NRCM in cell culture were exposed to preconditioned media of DCM-IgG treated NRCM. Hypertrophy response was confirmed by BNP mRNA expression measurements. Treatment of control NRCM by preconditioned media of DCM-IgG treated NRCM resulted in a significant increase in BNP mRNA expression, a marker for myocyte hypertrophy. Antibody based specific neutralization of VEGF signaling by inhibition of VEGFR-2 and neutralization of VEGF in the cell culture supernatant reversed the observed increase in BNP mRNA to baseline levels. *$p < 0.05$ vs. HC-IgG, #$p < 0.05$ vs. DCM-IgG. All $n = 3$ cell preparations. B, C) Induction of cell proliferation by VEGF Human coronary artery endothelial cells (HCAEC) and primary rat cardiac fibroblasts were incubated with equal volumes of preconditioned NRCM cell culture supernatant. Both cell types responded with a significant increase in cell proliferation. Antibody-based specific inhibition of VEGF signaling both by receptor-2 blockade or by VEGF neutralization confirmed that the observed increase in cell proliferation was induced by biologically active VEGF present in the cell culture supernatant. *$p < 0.05$ vs. HC-IgG, #$p < 0.05$ vs. DCM-IgG. All $n = 3$ cell preparations.

4.3. The role of VEGF signaling in cardiac remodeling

VEGF and its two receptors VEGFR-1 and VEGFR-2 play a pivotal role in angiogenesis under physiological or pathological circumstances and are involved in neuronal and myocardial development and morphogenesis [26]. Studies in animal models revealed that even small perturbations in VEGF levels affect cardiovascular development and embryonic survival [27,28]. Abnormal VEGF conditions may favor the initiation and progression of cardiac remodeling due to hypertrophy of cardiomyocytes [5,6]. Furthermore it appears of great importance that VEGF, which is released by cardiomyocytes enhances cardiac fibroblast proliferation in a paracrine manner [7]. A pathological proliferation of fibroblasts may favor the progression of myocardial fibrosis that might further trigger VEGF induction due to reduction in oxygen tension as a result of microcirculatory disorders. This vicious cycle might perpetuate the progression of pathological remodeling driven by an undirected and non-physiological VEGF release. This consideration is further enhanced by the finding that mechanical overload, an ubiquitous stimulus in DCM, also leads to an increase in VEGF expression by cardiomyocytes that is further enhanced by such autoantibodies.

The present study extends the knowledge about the possible link between autoimmune signaling in DCM and VEGF induction in cardiomyocytes. This novel finding may contribute to new pharmacological approaches to modulate myocardial remodeling in DCM on a molecular basis.

5. Limitations

The following limitations have to be considered a) The species-specific expression pattern and function of many proteins may differ substantially between rodent and human cells and between the conditions in-vitro and in-vivo. Therefore, the rodent in-vitro model used in this study is not completely transferable to the multiple mechanisms in DCM patients. b) The DCM-IgG concentrations chosen in this study are optimized for our in-vitro model of cultivated NRCM and are not necessarily representative for DCM in-vivo. c) The sequential relationship between the immune response with the release of autoantibodies and the development of DCM is rather unknown. d) VEGF polymorphisms with different degree of efficacy may occur under pathological circumstances and need to be further investigated in this scenario.

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Conflict of interest

none declared.

Disclosures

None.

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III

RATE AND IRREGULARITY OF ELECTRICAL ACTIVATION DURING ATRIAL FIBRILLATION EFFECT MYOCARDIAL NGF EXPRESSION VIA DIFFERENT SIGNALLING ROUTES.

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Rate and irregularity of electrical activation during atrial fibrillation affect myocardial NGF expression via different signalling routes

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ABSTRACT

An irregular ventricular response during atrial fibrillation (AF) has been shown to mediate an increase in sympathetic nerve activity in human subjects. The molecular mechanisms remain unclear. This study aimed to investigate the impact of rate and irregularity on nerve growth factor (NGF) expression in cardiomyocytes, since NGF is known to be the main contributor to cardiac sympathetic innervation density. Cell cultures of neonatal rat ventricular myocytes were electrically stimulated for 48 h with increasing rates (0, 5 and 50 Hz) and irregularity (standard deviation (SD) = 5%, 25% and 50% of mean cycle length). Furthermore, we analyzed the calcineurin-NFAT and the endothelin-1 signalling pathways as possible contributors to NGF regulation during arrhythmic stimulation. We found that the increase of NGF expression reached its maximum at the irregularity of 25% SD by 5 Hz (NGF: 5 Hz 0% SD = 1 vs. 5 Hz 25% SD = 1.57, $P<0.05$). Specific blockade of the ET-A receptor by BQ123 could abolish this NGF increase (NGF: 5 Hz 25% SD + BQ123 = 0.66, $P<0.05$). High frequency electrical field stimulation (HFES) with 50 Hz decreased the NGF expression in a significant manner (NGF: 50 Hz = 0.55, $P<0.05$). Inhibition of calcineurin-NFAT signalling with cyclosporine-A or 11R-VIVIT abolished the HFES induced NGF down-regulation (NGF: 50 Hz + CsA = 1.14, $P<0.05$). In summary, this study reveals different signalling routes of NGF expression in cardiomyocytes exposed to increasing rates and irregularity. Whether this translates into different degrees of NGF expression and possibly neural sympathetic growth in various forms of ventricular rate control during AF remains to be elucidated in further studies.

1. Introduction

Atrial fibrillation (AF) is associated with a 1.5- to 1.9-fold mortality risk after adjustment for preexisting cardiovascular conditions [1]. Many factors may contribute to this worse outcome like arterial embolism or hemodynamic deterioration. Recently, an irregular ventricular response during AF has been shown to increase sympathetic nerve activity in vivo [2,3]. This raises the hypothesis whether the irregular activation of cardiomyocytes may affect neural growth and activity.

Nerve growth factor (NGF) plays a key role in the structural and functional integrity of sympathetic neurons and regulates survival, differentiation and neurite outgrowth [4]. For example, in NGF knockout mice the volume of the sympathetic ganglia is remarkably reduced [5]. In contrast, over-expression of NGF in the heart results in cardiac hyperinnervation and hyperplasia of stellate ganglia neurons [6]. Up to now molecular mechanisms, which regulate cardiac NGF expression are not fully understoodd but endothelin-1 and calcineurin pathways seem to be critically involved: Ieda and colleagues first demonstrated that endothelin-1 up-regulates NGF expression in the rodent heart [7]. On the other hand, downregulation of myocardial NGF expression during cellular stretch is mediated via the calcineurin-NFAT signalling pathway [8].

In the present study, we investigated whether two key parameters of AF, the rapid electrical activation and the irregularity of electrical activation, affect NGF expression of cultured NRVM. In addition,

Abbreviations: ANP, Atrial natriuretic polypeptide; NGF, Nerve growth factor; ET-1, Endothelin-1; ET-A, Endothelin A receptor; GAP-43, Growth associated protein 43; Cn, Calcineurin; CsA, Cyclosporine A; NFAT, Nuclear factor of activated T-cells; NRVM, Neonatal rat ventricular myocytes; SCG, Superior cervical ganglia; HFES, High frequency electrical field stimulation; IF, Immunocytofluorescence.

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molecular signalling routes possibly involved in AF modulated NGF expression were analyzed.

2. Materials and methods

2.1. Cell cultures of neonatal rat ventricular myocytes (NRVM) and superior cervical ganglia (SCG) and application of electrical field stimulation

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). Cells were isolated and cultured as described previously [9–11]. "n" represents the number of cell preparations. For each NRVM cell preparation we used in average 10–15 neonatal rats. For each SCG cell preparation we used in average 20–25 neonatal rats. Three days after cell isolation serum containing medium was changed to serum-free medium and thereafter cell cultures of NRVM were paced for 48 h with increasing rates (0, 5 and 50 Hz). To analyze the effect of irregularity alone irregular stimuli (standard deviation (SD) = 5%, 25% and 50% of mean cycle length) were applied at each given frequency of stimulation with the C-PaceEP external pacing system (IonOptix, The Netherlands). The heart frequency of rats and humans is in a relation of 5–10:1 (depending on age with higher rates in neonatal rats). Thus, we performed a 5 Hz (300 bpm) electrical stimulation as an experimental equivalent to SR in humans. All experiments were performed at 2-fold myocytes threshold intensity with 2 V/cm at an impulse duration time of 1 ms as described previously [12]. To further assess the effect of NGF on neuronal growth the medium of stimulated myocytes was collected after 48 h of pacing and were incubated with SCG cell cultures for further 48 h.

2.2. RNA preparation and first-strand cDNA synthesis

Total RNA was extracted from NRVM using the Qiagen RNeasy Mini Kit and following the manufacturer's instructions. A total of 1 μg of RNA were reverse transcribed using random hexamers from the Fermentas First strand cDNA Synthesis Kit (#K1622).

2.3. Quantitative real time reverse transcription-PCR

Real-time PCR was performed in 96-well plates on the Step-One plus Sequence Detection System (ABI) as described previously [13]. Data were collected with instrument spectral compensation by Applied Biosystems SDS 1.2.3 software.

2.4. Primers and probes for quantitative real-time reverse transcription-PCR

PCR primers and fluorogenic probes for the target gene and the endogenous control were purchased as Assays-On-Demand (Applied Biosystems, Foster City, CA). The assay numbers for the endogenous control and target genes were as follows: Rn00560865_m1 (beta-2 microglobulin), Rn01533872_m1 (Ngfb), Rn00561661_m1 (Nppa).

2.5. ELISA for NGF

For NGF ELISA the conditioned media was collected at 0, 24 and 48 h after exposure to HFES and samples were assayed by a NGF kit from R&D Systems (Minneapolis, USA) according to the manufacturer´s instructions.

2.6. Western blot

Cells were homogenized in lysis buffer containing 128 mM Tris–HCl (pH 7.6), 4.6% SDS and 10% glycerol. Cell lysates were cleared by centrifugation and equal amounts of proteins were subjected to electrophoretic separation through a 10% SDS-polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20 (PBST). Blots were washed in PBST and incubated first with a primary (90 min) and thereafter with a secondary horseradish peroxidase (HRP)-conjugated antibody (90 min, GE Healthcare, UK). Finally, the ECL Advance Western Blotting Detection Kit (GE Healthcare Life Sciences, München, Germany) was used to visualize the bands.

2.7. Drugs and antibodies

Depending on the drug protocol 1×10^{-7} mol/L of BQ123, 1×10^{-6} mol/L of Cyclosporin A (CsA) or 1×10^{-6} mol/L of 11R-VIVIT (VIV) (a cell permeable version of NFAT inhibitor) was added at the initiation of electrical stimulation [14]. CsA and BQ123 were purchased from Sigma-Aldrich (Steinheim, Germany), 11R-VIVIT was purchased from Calbiochem (Darmstadt, Germany).

Primary antibodies for Western blot and immunocytofluorescence: rabbit anti-GAPDH (Cell Signalling), rabbit anti-GAP-43 (Santa Cruz), rabbit anti-NGF (Santa Cruz), mouse anti-endothelin-1 (acris-antibodies), rabbit anti-calcineurinA + B (alpha-diagnostics), goat anti-NFATc3 (Santa Cruz), rabbit anti-phosphorylated NFATc3 (Abcam) and rabbit anti-ANP (Santa Cruz).

2.8. Immunocytofluorescence

Cells were fixed in 4% paraformaldehyde, permeabilized with TritonX-100 and blocked with 1% BSA. Cells were incubated with the primary antibody overnight at 4 °C and then with a secondary antibody coupled to Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen). Nuclei were stained with DAPI. To visualize fluorescence signals and to measure the relative fluorescence profiles the Axiovert-200 M microscope and the AxioVision Rel.4.5 software from Zeiss were used. The myocytes were interactively contoured. The colour values were exported as tabular data. The mean values of all microscopic images were obtained. Finally, all values were calculated as relative values and were compared to each other by statistical analysis. Cells were normalized to cell bodies.

2.9. Measurement of cell surface area

Cultured cardiomyocytes were fixed and stained as described above. Quantification of cell surface area was performed by measuring 300 cardiomyocytes in 30 fields examined in each group, and the average value was used for statistical analysis. Cell surface data are presented as relative values.

2.10. Statistical analysis

All values are expressed as mean ± SEM. Comparisons of two groups were performed by Student's *t*-test and multiple groups by 1-way ANOVA followed by Bonferroni's post-hoc test. Values of $P<0.05$ were considered statistically significant. "n" represents the number of cell preparations.

3. Results

3.1. Arrhythmic stimulation increases NGF expression in ventricular myocytes

We first analyzed NGF mRNA expression in cardiomyocytes exposed to increasing frequency (0, 5 and 50 Hz) and irregular stimulation (5, 25 and 50% SD). At a constant rate of 5 Hz, we observed a slight but insignificant increase of NGF expression at a low degree of irregularity (5% SD). The increase in NGF mRNA expression became significant as irregularity rose to 25% SD (Fig. 1A). During irregular stimulation with 50% SD NGF

mRNA expression returned to control levels (Fig. 1A). By contrast, at a constant rate of 50 Hz NGF mRNA expression decreased significantly independent of the applied irregularity (Fig. 1B). Variability up to 50% SD remained without effect during stimulation with 50 Hz (Fig. 1B).

Specific pharmacological inhibition of the endothelin-A receptor (ET-A) with BQ123 completely prevented the NGF mRNA increase observed during electrical stimulation with 5 Hz at 25% SD (Fig. 1C). Pretreatment of irregular stimulated cells with cyclosporine-A (CsA) or 11R-Vivit (a cell permeable NFAT inhibitor) remained without effect (Fig. 1C). Likewise, pretreatment with BQ123 remained without effect on HFES (50 Hz) induced NGF mRNA down-regulation (Fig. 1D). CsA or 11R-Vivit abolished the HFES induced NGF mRNA down-regulation significantly (Fig. 1D).

Next, we performed highly sensitive ELISA experiments with cardiomyocyte cell-conditioned medium to analyze the secretion of NGF in the cell culture supernatant. We found that the increase in NGF mRNA expression by 5 Hz at 25% SD was paralleled by higher NGF levels in the cell culture supernatant (Fig. 2A). Pretreatment with BQ123 blunted this NGF increase (Fig. 2C). On the other hand, HFES with 50 Hz significantly decreased the amount of NGF in the cell culture supernatant (Fig. 2 B). This effect was not altered by irregularity. Pretreatment with CsA or 11R-Vivit normalized the HFES induced NGF down-regulation almost to control levels (Fig. 2D). The ELISA data could be confirmed by Western blot experiments. Arrhythmic stimulation significantly increased NGF protein levels in cardiomyocytes. This effect could be abolished by BQ123 (Fig. 3 A). On the other hand, HFES contributed to a decrease of NGF in cardiomyocytes, which could be prevented by pretreatment with CsA or 11R-Vivit (Fig. 3B).

3.2. Activation of either ET-1 or Cn-NFAT pathway depends on rate and irregularity

The expression of ET-1 protein increased significantly in 5 Hz stimulated cardiomyocytes at 25% SD as compared to 5 Hz stimulation without variability (Fig. 3C). A higher variability up to 50% SD remained without significant effect on ET-1 expression as compared to 5 Hz. On the other hand, electrical stimulation by 50 Hz resulted in a significant up-regulation of Cn protein expression (Figs. 3D and 4A) accompanied by a significant NFATc3 nuclear translocation (Fig. 4C). Additionally, Western blot analyzes for phosphorylated NFATc3 showed a significant decrease due to electrical stimulation by 50 Hz (Fig. 4D) indicating an activation of the Cn-NFAT signalling pathway.

As shown by the depicted Immunocytofluorescence images 50 Hz stimulated cells demonstrate a lager cell body confirmed by cell surface area measurements, indicating hypertrophy of cardiomyocytes (Fig. 4B). The up-regulation of ET-1 and subsequently NGF by arrhythmic pacing was not paralleled by an increase in ANP mRNA and protein expression, whereas HFES with up-regulation of the Cn-NFAT pathway and concomitantly NGF down-regulation resulted in a significant increase in ANP mRNA and protein expression, a marker for hypertrophy of cardiomyocytes (Fig. 6A–B).

Together, these data demonstrate that cardiomyocytes respond to increasing frequency or irregular stimulation with two different signalling routes to control the local NGF milieu.

3.3. The NGF increase during arrhythmic stimulation returns to control levels after cessation of the arrhythmia

Fig. 5A depicts cardiomyocytes in cell culture, double stained for troponin-t (red) and NGF (green). This image demonstrates the abundance of NGF that is expressed by ventricular myocytes. To analyze, whether the effect of arrhythmic pacing on NGF expression in cardiomyocytes is sustained after cessation of the irregular stimulation, we performed further experiments using an altered stimulation program. After an initially arrhythmic stimulation with 5 Hz at 25% SD for the first 24 h, we switched off the irregular stimulation and continued at 5 Hz without the variability of 25% SD for further 24 h. We found that the initial (first 24 h) increase in NGF expression under conditions of

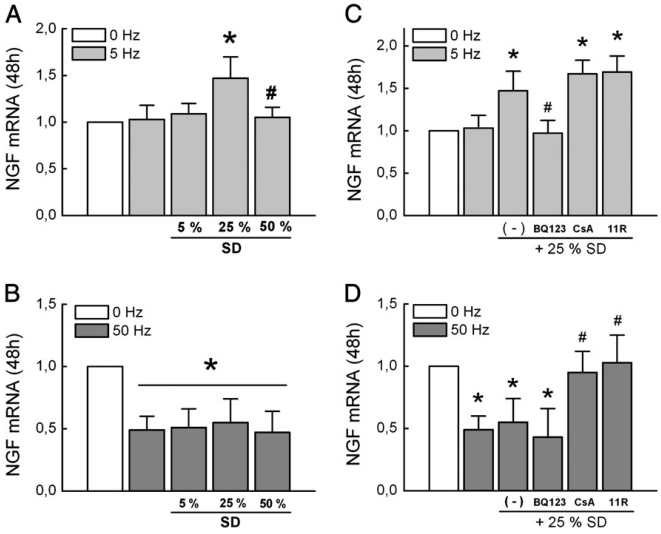

Fig. 1. Expression of NGF in ventricular myocytes depends on stimulation rate and irregularity. Panels A and C demonstrate that an irregular stimulation with 25% SD significantly increases NGF mRNA expression in ventricular myocytes. ET-A receptor blockade by BQ123 is effective in preventing this NGF increase, whereas inhibition of the calcineurin-NFAT pathway remains without effect on the NGF down-regulation induced by irregular stimulation. As frequency rises, NGF mRNA expression decreases in a significant manner (B). Whereas inhibition of the calcineurin-NFAT pathway is effective in preventing HFES induced NGF downregulation, blockade of the ET-A receptor by BQ123 remains without effect (Fig. 1D). All PCR data are derived from at least $n = 3$ experiments. *$P < 0.05$ vs. 0 Hz. #$P < 0.05$ vs. 5 Hz + 25% SD or 50 Hz.

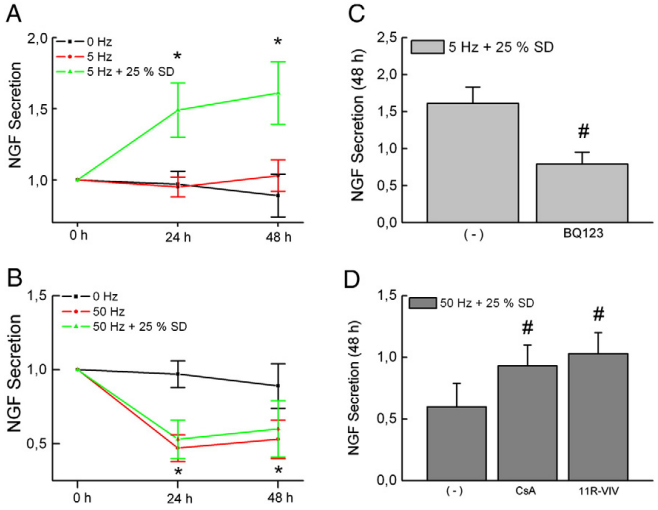

Fig. 2. NGF mRNA increase is accompanied by a higher NGF secretion into the cell culture supernatant. ELISA experiments revealed higher NGF levels in the cell culture supernatant as irregularity rose to 25% SD at a constant rate of 5 Hz. These experiments also revealed time dependency. NGF increase showed slightly higher values at 48 h as compared to 24 h (A). Application of BQ123 (an ET-A receptor inhibitor), prevented the NGF increase significantly (C). HFES with 50 Hz over a stimulation period of 48 h decreased NGF levels in the cell culture supernatant significantly. This response peaked at 24 h with a slight increase after 48 h of HFES stimulation. Irregularity with 25% SD showed no significant effect on NGF secretion (B). Pretreatment with CsA or 11R-Vivit prevented the HFES induced NGF decrease (D). All ELISA data are derived from at least $n=3$ experiments. $^{*}P<0.05$ vs. 0 Hz. $\#P<0.05$ vs. 5 Hz + 25 SD or 50 Hz + 25% SD.

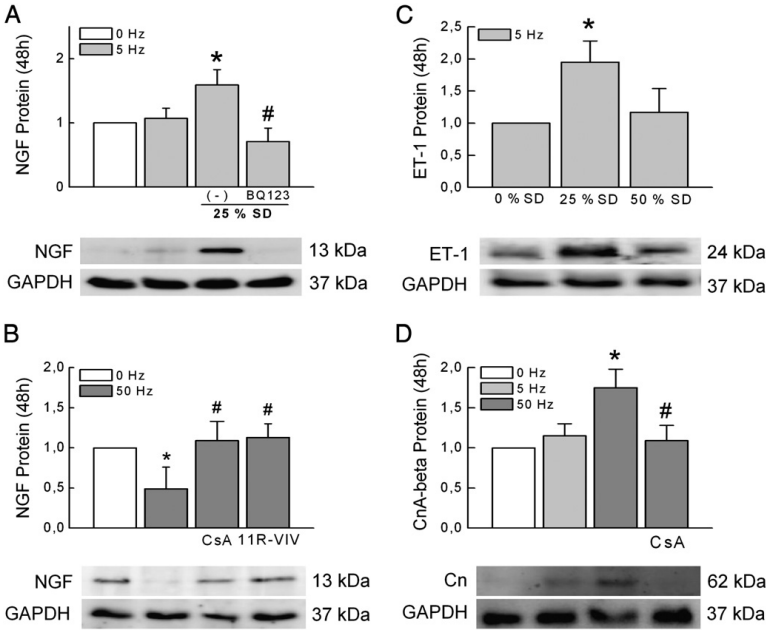

Fig. 3. Representative Western blots depicting myocytes protein expression of NGF, ET-1 and Cn. At a constant rate of 5 Hz, NGF protein levels showed a significant increase as irregularity rose to 25% SD (A). ET-A blockade by BQ123 prevented the NGF increase to basic values. Conversely, HFES with 50 Hz led to a decrease in NGF protein levels almost to 50% of the levels found in control cells (B). CsA or 11RVivit prevented the HFES induced NGF down-regulation to control levels. ET-1 expression showed the highest increase at 5 Hz with 25% SD. Higher levels of irregularity were associated with a return of the ET-1 expression to control levels (C). Cn expression increased at 50 Hz almost 1.5 fold as compared to 0 Hz. CsA inhibited this increase significantly (D). All WB data are derived from at least $n=5$ cell preparations. $^{*}P<0.05$ vs. 0 Hz or 5 Hz. $\#P<0.05$ vs. 5 Hz + 25 SD or 50 Hz.

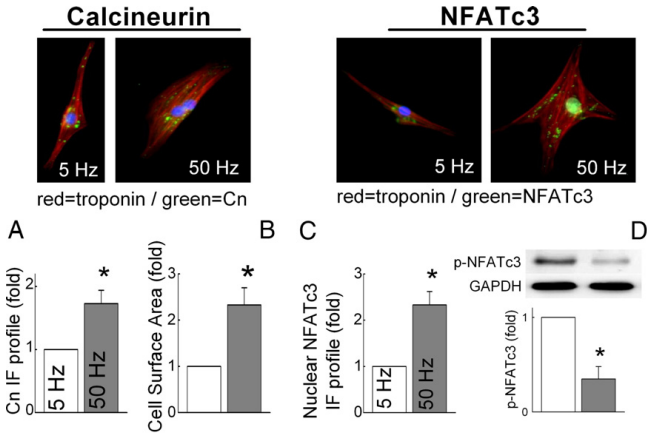

Fig. 4. Immunocytofluorescence (IF) images depicting Cn and NFATc3 nuclear translocation expression in NRVM. Cn-NFAT activation was analyzed by immunocytofluorescence and Western blot experiments of 5 Hz and 50 Hz stimulated cells. Cn IF profile increased at 50 Hz stimulation (A). Moreover, 50 Hz stimulated cells demonstrate a lager cell body confirmed by cell surface area measurements, indicating hypertrophy of cardiomyocytes (B). In parallel, NFATc3 translocation also showed a significant increase at 50 Hz stimulation (C). For each analysis, 30 cells from at least $n = 3$ cell preparations were examined. In addition, Western blotting of phosphorylated NFATc3 showed a significant decrease at 50 Hz stimulation indicating an activation of the Cn-NFAT pathway (D). *$P<0.05$ vs. 5 Hz.

arrhythmic pacing returned almost to baseline levels after termination of the irregular stimulation (Fig. 5B).

3.4. Increased NGF levels due to arrhythmic stimulation are sufficient to promote neurite outgrowth in sympathetic neurons

To determine whether the increase in cardiomyocyte NGF expression and secretion in terms of irregular stimulation is sufficient to induce neurite outgrowth in sympathetic neurons, primary cultures of sympathetic neurons were incubated for 48 h with cardiomyocyte cell-conditioned medium that were collected after 48 h of electrical stimulation. The higher amount in NGF protein levels, resulting from arrhythmic stimulation contributed to an increase in neurite outgrowth in a significant manner as shown by Immunocytofluorescence and Western blot experiments for GAP-43 protein expression, an established marker for neuronal growth (Fig. 5C, D).

4. Discussion

The major findings of the present study are: a) Cardiomyocytes control their local NGF milieu under conditions of high rate or irregular stimulation by activating two different signalling routes. b) The ET-1 signalling pathway contributes to an increase in NGF expression due to irregular stimulation with a maximum at 25% SD, apparently without affecting cardiomyocyte hypertrophy. c) The Cn-NFAT signalling pathway significantly depresses NGF expression as frequency

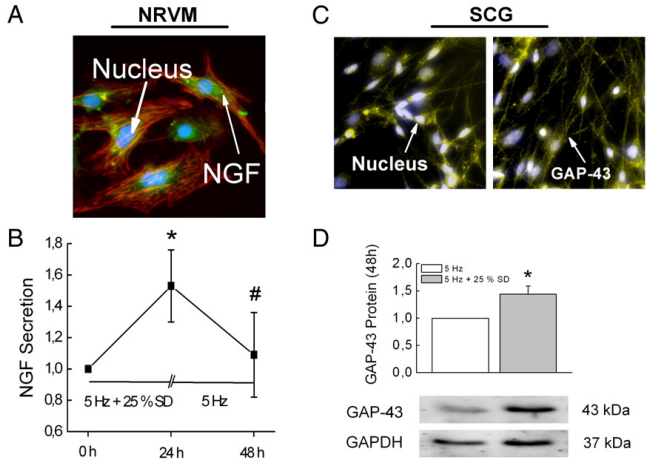

Fig. 5. IF images showing NRVM with NGF expression and SCG cell cultures with GAP-43 expression. Panel A demonstrates the abundance of NGF expressed by ventricular myocytes. The NGF increase due to arrhythmic stimulation returns to control levels after the arrhythmia is switched off (B). The levels of NGF in the cell culture supernatant of 5 Hz + 25% SD stimulated NRVM cells are sufficient to promote neurite outgrowth of cultured sympathetic neurons (C, D). ELISA and WB data are derived from at least $n = 3$ cell preparations. *$P<0.05$ vs. 5 Hz + 25% SD at 0 h (ELISA) or 5 Hz at 48 h (WB). #$P<0.05$ vs. 5 Hz + 25 SD at 24 h (ELISA).

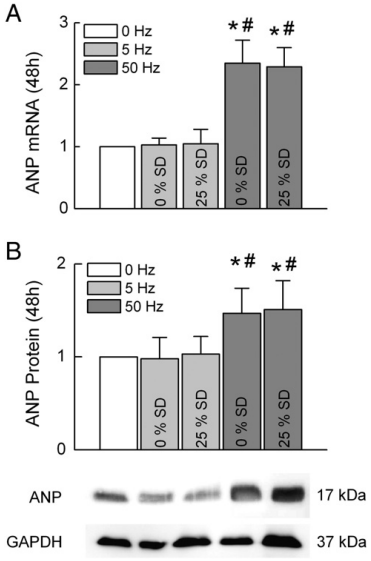

Fig. 6. ANP expression of NRVM exposed to increasing frequency and variability stimulation. We observed no increase in ANP mRNA expression at a stimulation frequency of 5 Hz. Variability up to 25% SD remained also without effect on ANP mRNA expression. Conversely, HFES with 50 Hz increased ANP mRNA expression almost two fold, without being affected by the most effective variability of 25% SD (A). The gene expression data could be confirmed by Western blotting (B). *$P<0.05$ vs. 0 Hz. #$P<0.05$ vs. 5 Hz.

rises to 50 Hz without being affected by irregular stimulation. Hypertrophy cascades, as indicated by ANP up-regulation accompany the activation of the Cn-NFAT pathway.

4.1. Rate and irregularity activates two different signalling routes in cardiomyocytes

The present study analyzed the influence of rapid electrical activation of cultured ventricular myocytes with different degrees of rate and irregularity on the expression and secretion of NGF. Furthermore, we investigated the ET-1 and the Cn-NFAT pathway as possible contributors to NGF regulation. It seems as if the single cardiomyocyte possess the genetic capacity to respond to both stimuli with contrarily cellular programs. NRVM increased at a constant rate of 5 Hz the cellular NGF production with a maximum at 25% SD. A higher variability (50% SD) remained without effect on cellular NGF expression. The ET-1 cascade seems to play an important role for this phenomenon while the calcineurin pathway was not involved. Likewise, no signs of cellular hypertrophy were observed. On the other hand, HFES at regular rate with 50 Hz decreased the expression of NGF in a significant manner accompanied by cellular hypertrophy. Since HFES promotes intracellular calcium overload this may have contributed to activation of the Cn-NFAT pathway with subsequent downregulation of NGF expression. These results throw a completely new light on the understanding of irregular heartbeat. The discussion about rate vs. rhythm control does not seem to be fully cleared up.

4.2. ET-1, NGF and lethal arrhythmias

ET-1 serves several functions in cardiac myocytes. It regulates the movements and intracellular concentrations of ions, affecting contractile properties [15]. It is involved in cellular hypertrophy cascades [16] and plays a crucial role in pressure overload signalling routes in ventricular and atrial cardiomyocytes [17,18]. In addition, Ieda and colleagues identified ET-1 as an NGF up-regulating peptide in the rodent heart [7]. NGF is not only the major neurotrophic factor for the survival and differentiation of sympathetic neurons, it even functions as a pro-survival factor for cardiomyocytes that regulates apoptosis [19] and plays a pivotal role in cardiac repair following myocardial infarction [20]. Thus, NGF seems to be a double-edged sword, since pathological NGF regulation is also involved in nerve sprouting and sudden cardiac death [21]. The mechanisms by which cardiac NGF expression is up- or downregulated are poorly understood despite its obvious crucial role in the neurophysiology of the heart. Recently, our group could show that mechanical stretch, an ubiquitous stimulus in cardiac tissue contributes to hypertrophy and NGF downregulation in cardiomyocytes via the Cn-NFAT pathway [8]. ET-1 induction in cardiomyocytes due to mechanical stretch has also been shown to be involved in hypertrophy cascades [22]. The cellular balance and interactions between both the ET-1 and the Cn-NFAT pathway in NGF and hypertrophy regulation during physiological or pathological circumstances still remains to be characterized. With the present study we could demonstrate that cardiomyocytes, which were electrically paced with different rates and irregularity over a period of 48 h, activated either the ET-1 or the Cn-NFAT pathway depending on the stimulation protocol. While the ET-1 cascade induced a NGF up-regulation to perform sympathetic nerve growth as a response to arrhythmic stimulation, the Cn-NFAT pathway was activated in terms of high frequency electrical field stimulation (HFES) and resulted in NGF down-regulation and hypertrophy signalling cascades. Whether this translates into different degrees of NGF expression and possibly neural sympathetic growth in various patterns of ventricular rate control during human AF remains to be elucidated in further studies.

5. Limitations

AF generally occurs in the elderly. Thus, our in-vitro model of NRVM differs substantially from the situation in human subjects. The expression pattern of many proteins may vary significantly between the cells used by us and adult rats or human myocytes. Thus, our isolated myocytes do not provide the full picture of activated signalling routes involved in NGF regulation induced by HFES or arrhythmic stimulation. The reason to use NRVM for an in-vitro disease model that generally occurs in the elderly is that NRVM are a well-characterized tool to investigate fundamentals in cellular signalling. Furthermore, we did not analyze the activation balance between ET-1 and the Cn-NFAT pathway during HFES, since the regulation in both cascades seems to be regulated by similar stimuli such as mechanical stretch or HFES. Electrical stimulation was performed at twice the diastolic pacing threshold. Thus, the electrical field applied is considerably higher than during spontaneous AF, when cells are excited via spontaneous wave propagation via gap junctions and excitatory potentials.

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Conflict of interest

None declared.

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IV

MECHANICAL STRETCH OF SYMPATHETIC NEURONS INDUCES VEGF EXPRESSION VIA A NGF AND CNTF SIGNALING PATHWAY.

Saygili E, Pekassa M, Saygili E, Rackauskas G, Hommes D, Noor-Ebad F, Gemein C, Zink MD, Schwinger RH, Weis J, Marx N, Schauerte P, Rana OR.

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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 Sprangue 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

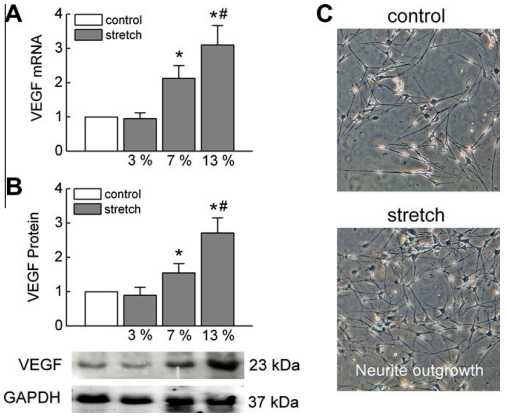

Fig. 1. Mechanical stretch of SN increases VEGF mRNA and protein expression in a time and dose depended manner. SN were exposed to a gradual increase of mechanical stretch over a time period of 72 h. (A) Real-time PCR showed a time and dose depended increase in VEGF mRNA expression by stretch up to 13%. (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

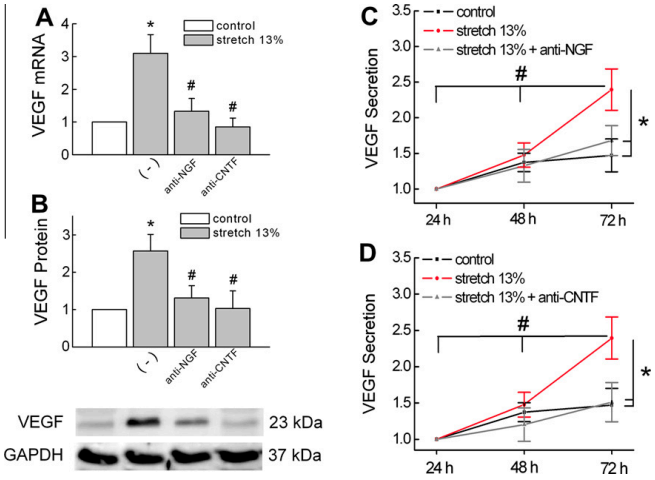

Fig. 2. Stretch-induced neuronal VEGF augmentation is mediated by NGF or CNTF. SN were exposed to a gradual increase of mechanical stretch over a time period of 72 h in the presence or absence of NGF or CNTF neutralizing antibodies. (A) On mRNA level NGF or CNTF neutralization blunted the stretch-induced 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.

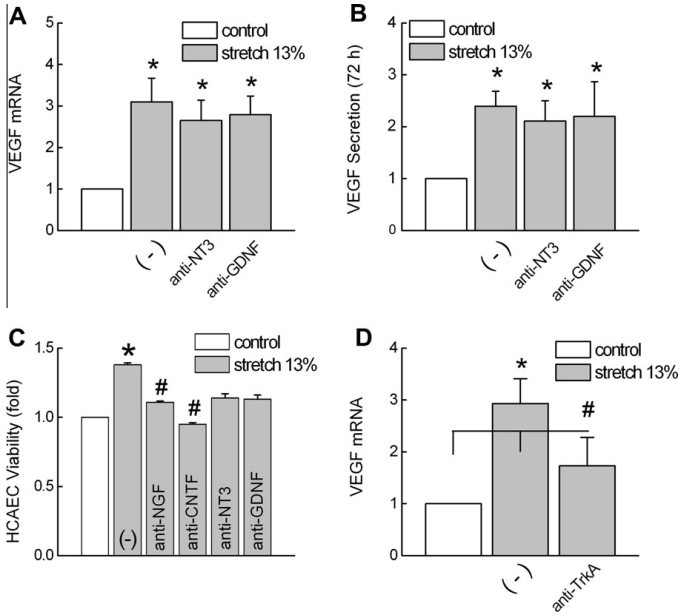

Fig. 3. NT-3 or GDNF remained without significant effect on stretch-induced neuronal VEGF expression. (A-B) ELISA data are presented in panel diagrams with samples from 72 h. Neither NT-3 nor GDNF could prevent the stretch-induced VEGF increase on mRNA or protein level. (C) To demonstrate the biological 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

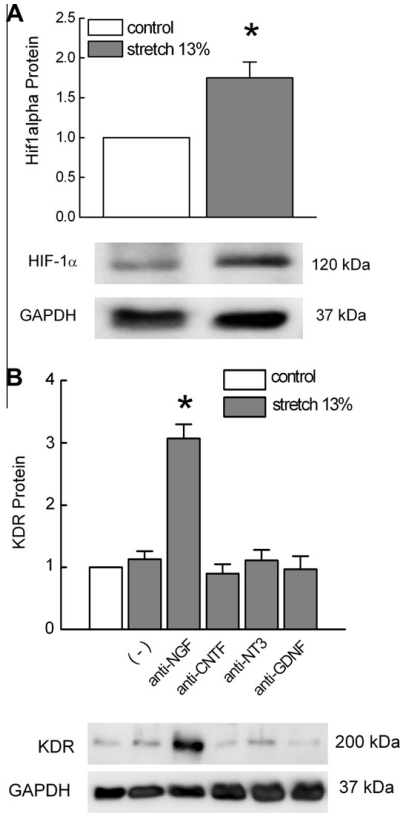

Fig. 4. The role of stretch and NGF signaling on neuronal Hif-1 α and KDR expression. (A) Stretch by 13% contributed to an increase in neuronal Hif-1α expression, (B) but remained without a significant effect on neuronal KDR expression. NGF neutralization increased neuronal KDR expression significantly, 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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