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Cardiac microvascular endothelial cells express and release nerve growth factor but not fibroblast growth factor-2

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Abstract Endothelial cells (ECs) from different vascular beds not only display common characteristics but are also quite heterogeneous in terms of expression and secretion of neuro-angiogenic factors, which may help explain some of their distinct physiological roles. We investigated by RT-PCR the gene expression, by PC12 bioassay the neurotropic activity, and by ELISAs the levels of NGF and FGF-2 using conditioned medium collected from cultures of ECs derived from myocardial and cerebral capillaries. While NGF was expressed and released by both cell types, FGF-2 was expressed and released solely by the brain but not heart ECs. Oxygen-glucose deprivation (ischemic) insult blocked NGF secretion from heart and brain ECs and inhibited by 70% the secretion of FGF-2 from brain ECs. We propose that the differential expression of NGF and FGF-2 in heart and brain EC cultures reflect heterogeneity on demand of the microcapillary components and the surrounding microenvironment for a proper tissue-specific homeostasis.

Keywords Brain · Endothelial heterogeneity · FGF-2 · Heart · NGF

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Introduction

Endothelial cells (ECs) synthesize and release angiogenic factors such as fibroblast growth factor-2 (FGF-2) (Schweigerer et al. 1987) and vascular endothelial growth factor (VEGF) (Semenza 2003), particularly under pathophysiological conditions of inflammation, hypoxia, and oxidative stress (Fraisl et al. 2009). These factors, together with neurotrophins, through paracrine or autocrine signaling, regulate both ECs survival and angiogenesis and also participate in the cross-talk between the cardiovascular and the nervous system (Lazarovici et al. 2006). To date, very few studies have addressed the heterogeneous pattern of expression of FGF-2, a typical angiogenic factor and nerve growth factor (NGF), a prototypic neurotrophin, in ECs derived from distinct microvascular beds.

The neurotrophins NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are an important family of growth factors that promote survival, differentiation, proliferation, and neuroprotection of sympathetic and sensory neurons innervating the heart (Reichardt 2006). Recently, NGF and other neurotrophins have been recognized as important factors in angiogenesis (Lazarovici et al. 2006) and cardiac homeostasis (Lommatzsch et al. 2005). These factors signal by activation of tropomyosin-related kinase receptors (Trk), which are a family of tyrosine kinase receptors found in many tissues including the heart (Caporali and Emanueli 2009)

and inhibited by the partial antagonist K252a (Koizumi et al. 1988), the most-characterized pharmacological tool to inhibit and to study Trk receptors. It has been previously shown that the heart expresses NGF (Korsching and Thoenen 1983; 1988), and its level is reduced in heart failure both in experimental animal models as well as in patients (Kaye et al. 2000). Similarly, the levels of NGF are downregulated in acute coronary syndrome (Manni et al. 2005) and congestive heart failure (Kaye et al. 2000). NGF can protect the failing heart against post-ischemic dysfunction of sympathetic coronary innervation (Abe et al. 1997; Ieda and Fukuda 2009) and, on the other hand, is able to augment sympathetic nerve sprouting increasing the probability of ventricular arrhythmia (Cao et al. 2000). Taken together, these studies indicate the important role of NGF in heart physiology and pathology. However, with all this knowledge, the specific cellular source or regulation of NGF in the heart is not yet understood.

In this study, we compared expression and secretion of FGF-2 and NGF in vitro in microvascular EC lines isolated from distinct anatomical locations and found an increased expression of NGF in the heart ECs as compared to ECs from the brain. Furthermore, expression and secretion of FGF-2 was restricted to the brain-derived microvascular ECs. We propose that the differential expression of these two growth factors reflects an increased local demand of the heart microcapillary environment for NGF, which may be necessary for a proper cardiac homeostasis, in addition to and above that supplied locally by the cardiac myocytes (Ieda and Fukuda 2009).

Materials and Methods

Materials. Mouse beta-NGF was purchased from Alomone Labs (Jerusalem, Israel), and FGF-2 was purchased from Peprotech (Rehovot, Israel). K252a was kindly provided by Fermentek (Jerusalem, Israel).

Endothelial cells. Four different types of microvascular endothelial cell lines (myocardium-derived, mouse MyEnd (Burek and Forster 2009) and rat RHE-A (Obermeyer et al. 2003) and mouse brain-derived, cEnd (Forster et al. 2005) and bEnd.3 (from ATCC, Manassas, VA) (Omidi et al. 2003)) were cultured at similar conditions in 75-cm^2 tissue culture flasks (Nunc, Denmark) coated with collagen IV (BD, Franklin Lakes, NJ). The ECs were tested routinely for expression of endothelial markers such as VE-cadherin and occludin. The growth medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mg/ml glucose, 10% fetal calf serum, 2 mM l-glutamine, 200 μM Na-pyruvate, penicillin 10,000 U/ml, and streptomycin 100 μg/ml (Beit Haemek, Afula, Israel). The cell cultures were maintained at 37°C in a humidified incubator in mixture of 5% CO_2/95% air. The medium was changed every second day. Confluent flasks were split at a 1:10 ratio by trypsinization of the cultures with 0.25% trypsin solution (Beit Haemek, Afula, Israel). In some instances, upon reaching 90% confluence, the spent growth medium was replaced with fresh, serum-free growth medium, and the cells were cultured for another 48 h to generate conditioned medium (CM). Thereafter, the medium was collected and filtered through 0.2 μm cellulose acetate filter (Whatman, Germany). The filtered CM was kept at −20°C and was thawed prior to use. Upon collecting the CM, EC monolayers were trypsinized and the number of cells was counted in a hemocytometer in order to normalize the concentration of factors in the CM to the number of cells. All cell cultures were maintained and handled in a clean room 10,000 particles/m^3 according to ISO 7 standard.

PC12 bioassay. PC12 bioassay for measuring neurite extension was performed as previously described (Katzir et al. 2002). PC12 cultures (from ATCC) were tested routinely for expression of TrkA and response to NGF with extension of neurites. Briefly, the PC12 cultures were treated with different concentrations of CM for 2 d. Neuronal differentiation was quantified from images acquired with a Nikon Coolpix5000 camera mounted on a Nikon Eclipse TS100 microscope at ×200 magnification. To assess neuronal differentiation, total neurite length was quantified using the SigmaScanPro 5.0 program (Systat, Sweden). The experiments were performed in triplicates. In each well, three random chosen regions of interest were photographed. In each region of interest, 15–25 cells were measured; thus, at least 135 cells were measured for each treatment. The data are expressed as elongation factor (E), which is generated by dividing the total length of outgrowths from each cell in the region of interest by the cell diameter, with $E>2.0$ for true neurite extension (Katzir et al. 2002).

NGF and FGF-2 ELISA. Samples of media were collected from the various EC cultures grown under regular (normoxia) and oxygen-glucose deprivation (OGD) ischemic-like conditions. Concentrations of the growth factors in cell culture supernatants were determined by commercial ELISAs (Arien-Zakay et al. 2009), according to the manufacturer's instructions. FGF-2 was quantified using a FGF-2 ELISA Development Kit (PeproTech, Rocky Hill, NJ). The amount of FGF-2 in picogram per milliliter was calculated from a human recombinant FGF-2 standard curve, with a sensitivity of 62 pg/ml. According to the manufacturer, this kit is 100% cross-reactive with mouse and rat FGF-2 and exhibits less than 1% cross-reactivity with other FGF isoforms, even at 50 ng/ml. NGF levels were estimated using the Promega's

Emax® ImmunoAssay System (Promega, Madison, WI). The amount of NGF in picogram per milliliter was calculated from an NGF standard curve, with a sensitivity of 8 pg/ml. The anti-NGF polyclonal antibody used in the assay cross-reacts with mouse, rat, and human NGF and shows less than 3% cross-reactivity with other neurotrophic factors even at 10 ng/ml.

Oxygen-glucose deprivation. To induce the OGD insult, ECs were cultured in 12 wells plates for 48 h and introduced into a homemade ischemia chamber that has been described before (Tabakman et al. 2002). Oxygen deprivation (less than 1% O_2) was achieved by replacing the ambient atmosphere (21% O_2) with a mixture of 5% CO_2/95% N_2, which was humidified by passing the gas through a water-containing cylinder to prevent dehydration of the cell cultures The oxygen levels in the device rapidly (<5 min) dropped below 1% (anoxia) and stayed constant during the entire duration of an experiment, as monitored online using a digital oxygen analyzer (Hudson RCI, Germany) and by analyzing dissolved oxygen in the medium samples using Corning 178 blood gas analyzer (Abu Raya et al. 1993). The ischemia chamber was maintained at 37°C using circulating hot water. To generate glucose starvation, in addition to the hypoxic insult, the regular DMEM was aspirated immediately before initiation of OGD experiment, and the cultures were washed three times with glucose-free DMEM followed by addition of 1 ml of the same medium. Thereafter, the cultures were introduced into the ischemia chamber for 6–8 h. Immediately upon termination of OGD insult, the CM was collected and cell death was assessed by measuring LDH release (Tabakman et al. 2002). Control cultures were maintained in the incubator with glucose-containing DMEM at ambient oxygen levels.

RT-PCR. Total RNA was isolated from cell cultures using the SV total RNA isolation kit (Promega, Madison, WI), according to the manufacturer's instructions. The amount of isolated RNA and the quality of the RNA were assessed by measuring the absorbance at 260/280 nm. From the total RNA isolates, 1 μg was used for reverse transcription with the Reverse Transcription kit (Promega) according to the manufacturer's protocol. For the PCR reactions, GoTaq® Green Master Mix (Promega) was used. The reactions were carried out with 5 μg cDNA and 70 pmol each of sense and antisense primers in a final volume of 30 μl. All primers were generated and validated as previously described (Arien-Zakay et al. 2009). The following mouse-specific primer pairs were used: (1) NGF sense—TGC TGA ACC AAT AGC TGC C and antisense—ATC TCC AAC CCA CAC ACT GAC, product size 271 bp; (2) BDNF sense—AGC CTC CTC TGC TCT TTC TGC TGG A and antisense—CTT TTG TCT ATG CCC CTG CAG CCT T, product size 298 bp; (3) NT-3 sense—CCG GTG GTA GCC AAT AGA ACC and antisense—GCT GAG GAC TTG TCG GTC AC, product size 119 bp; (4) NT-4 sense—CTC CTG AGT GGG ACC TCT TG and antisense—CAC TCA CTG CAT CGC ACA C; product size 198 bp; (5) VEGF sense—TGC ACC CAC GAC AGA AGG GGA and antisense—TCA CCG CCT TGG CTT GTC ACA T, product size VEGF-A_{121} 360 bp, VEGF-A_{165} 492 bp, and VEGF-A_{189} 564 bp; (6) FGF-2 sense—CCC AAG CGG CTC TAC T and antisense—TTT ATA CTG CCC AGT TCG TTT, product size 318 bp; (7) β-actin sense—TCA TGA AGT GTG ACG TTG ACA TCC GT; antisense—CTT AGA AGC ATT TGC GGT GCA CGA TG, product size 285 bp. The PCRs were started by denaturation of the mixture for 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at 58°C (for FGF-2 and VEGF), 60°C (for NGF and NT-4), and 62°C (for NT-3), 2 min at 72°C and a final extension step of 7 min at 72°C. β-actin was used as a normalization control and started with denaturation of 2 min at 94°C followed by 35 amplification cycles of 30 s at 94°C, 1 min at 65°C, 2 min at 68°C, and a final extension step of 7 min at 68°C. The PCRs were performed using Mastercycler gradient instrument (Eppendorf, Germany). The PCR products were separated by electrophoresis (100 V for 40 min) in agarose gel (2%) containing ethidium bromide for UV visualization (Arien-Zakay et al. 2009).

Statistics. The results are presented as the mean ± SD of at least three independent experiments (n=9–15) and were evaluated using the InStat3 statistics program (GraphPad, La Jolla, CA). Statistically significant differences between experimental groups were determined by ANOVA with Bonferroni post hoc test with $p<0.05$ considered significant.

Results

Among the neurotrophins investigated mRNA transcripts of NGF, BDNF, NT-3, and NT-4 were present in microcapillary ECs derived from mouse heart and brain (Fig. 1). In addition, three different transcripts of VEGF, corresponding to VEGF-A 121, 165 and 189 were also evident in both ECs. By contrast, FGF-2 transcripts were detected only in mouse brain ECs, but not in the mouse heart-derived microcapillary ECs. Similarly, FGF-2 transcripts were absent in rat heart ECs (data not shown). In parallel, all four EC lines used in this study were found to express NGF and FGF-2 receptors using western blotting (Lecht et al., submitted). Moreover, treatment of the EC cultures with NGF or FGF-2 was able to induce phosphorylation of the

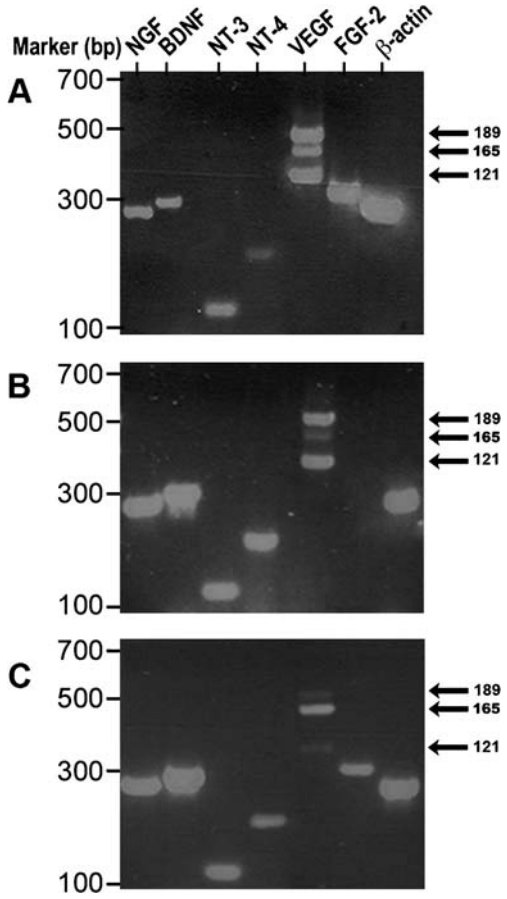

Figure 1. Differential expression of mRNA transcripts for angiogenic and neurotrophic factors in mouse brain ECs (*A*), mouse heart ECs (*B*) and total mouse brain extract (*C*) using RT-PCR. Total RNA of the different ECs was extracted; equal amounts of cDNA from each treatment were applied for RT-PCR using specific primers for each growth factor and carried out as described in "Materials and Methods". The products were separated by electrophoresis, stained, and photographed. The size marker is indicated on the left. VEGF-A isoform transcripts are indicated on the right. β-actin was used for reference purposes.

respective receptor and downstream signaling (Lecht et al., submitted).

In support of this finding, we measured the neurotopic activity of growth factors in the CM collected from all ECs using the neuronal PC12 bioassay (Katzir et al. 2002). Neurotrophic factors present in the CM of both cell types induced differentiation of the neuronal cells in a dose-dependent fashion (Fig. 2). This assay is rather selective; as seen in Fig. 3, only NGF or FGF-2 but not other neurotrophins or angiogenic factors will elicit neurotropic effects in PC12 cells. Since the PC12 bioassay cannot discriminate between neurotopic effects elicited by either NGF or FGF-2, we performed experiments with K252a, a partial antagonist of Trk receptors family compound, which inhibits in particular the activation of NGF/TrkA receptor but not that of FGF-2 receptors (Koizumi et al. 1988). For these studies, PC12 cells were treated with 50% CM of heart and brain ECs in the presence or absence of 100 nM K252a, and the neurotropic activity was evaluated 2 d later (Fig. 4*A*). Neurotropic activity of heart ECs-CM resulted with E factor of 2.7±0.2 significantly higher than that of untreated control cells (*E* of 0.8±0.5). This activity was inhibited by 37±7% in the presence of K252a (Fig. 4*A*), suggesting a specific presence of NGF in the CM, while the other half of the neurotropic effect is due to K252a partial agonist property. The neurotropic activity in the CM from brain ECs exhibited an increase of 3.4±0.2 folds in the neurotropic activity as compared to control. Importantly, this effect was enhanced by 47±5% in the presence of K252a, suggesting absence of bioactive NGF, while pointing towards FGF-2 as the one factor responsible for the majority of the neurotropic effects. Indeed, as shown before, mRNA for FGF-2 was present in the brain, but not in the heart ECs (Fig. 1*A*). The data presented in Fig. 4*B* strongly support this conclusion. K252a alone induced a slight neurotropic effect of 50% above basal levels, due to its properties as a partial agonist (Lazarovici et al. 1997). For NGF treatment, the inhibitory effect of K252a (at a nontoxic concentration of 100 nM) is about 65%. Although at higher concentrations, K252a completely inhibited the effects of NGF, it was slightly cytotoxic (data not shown). By contrast, the neurotropic activity of FGF-2 is augmented in the presence of K252a, indicating additive effects between FGF-2 and K252a, as also shown previously (Isono et al. 1994).

To further validate the differential expression of NGF and FGF-2 in heart and brain ECs, we quantified their amount using specific ELISAs under ambient oxygen pressure and following ischemic conditions of OGD (Fig. 5). Under ambient oxygen levels (normoxia), heart ECs secreted about 100 pg NGF/ml/10^6 cells during the first 6–8 h of culture, while brain ECs secreted about 30% of that. However, while cultures of brain ECs secreted about 190 pg FGF-2/ml/10^6 cells, CM of heart ECs contained less than 2 pg/ml/10^6 cells (value that is the detection limit of the commercial kit). These results support the findings from the PC12 cell bioassay indicating (1) higher levels of NGF in heart EC-CM than in brain EC-CM (Fig. 4) and (2) absence of FGF-2 (mRNA, protein).

Hypoxia (low level of oxygen) is a major pathophysiological condition for the induction of angiogenesis (Fraisl et al. 2009), which is known to be mediated by up-regulation and secretion of VEGF (Ferrara 2005) and/or FGF-2 (Calvani et al. 2006). However, the response of ECs to ischemia (low levels of both oxygen and glucose, i.e., OGD), particularly from different vascular beds in releasing growth factors such as FGF-2 and NGF, is still poorly understood. To investigate the effect of OGD on NGF and FGF-2 release, we exposed cultured heart and brain ECs to

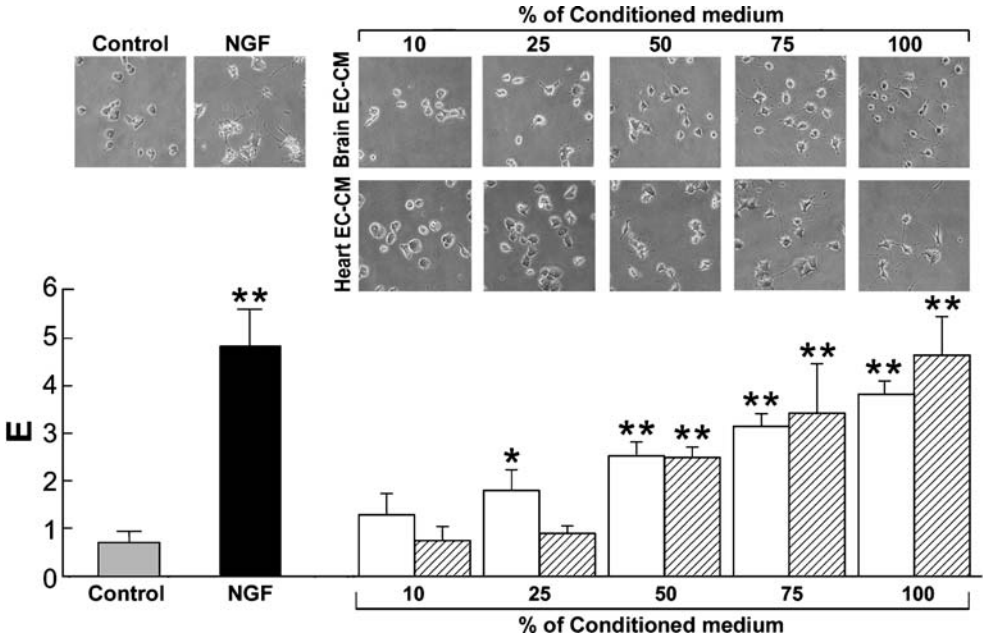

Figure 2. Dose-response measurements of neurotropic activity of brain and heart ECs CM using the PC12 bioassay. PC12 cells were treated with different concentration of brain ECs (*open bars*) and heart ECs (*stripped bars*) CM as described under "Materials and Methods". In parallel, PC12 cultures were treated with either 50 ng/ml NGF (*black bar*) or left untreated (*gray bar*). Upper part of the figure presents typical micrographs (magnification ×200) of the PC12 cells treated with different CMs indicating neuronal cells with neurite outgrowths with different length. The differentiation was estimated by elongation parameter (*E*) as described under "Materials and Methods". Data are mean ± SD of three different experiments (n=12–18). *p< 0.05 and **p<0.01 vs. control.

OGD for a period of 6–8 h insult and measured the levels of these growth factors using specific ELISAs (Fig. 5). The OGD insult suppressed the release of NGF from both heart and brain ECs and inhibited by 70±2% the release of FGF-2 from brain ECs while in heart EC-CM the level of FGF-2 remained less than 2 pg/ml/10^6 cells, i.e., below the threshold of detection of the ELISA kit. These results indicate that a strong insult such as ischemia-like OGD insult caused a significant reduction in the ability of cultured heart and brain ECs to secrete NGF and FGF-2, in contrast to hypoxia that induces increased secretion of NGF (Kim et al. 2004) and FGF-2 (Calvani et al. 2006).

Discussion

In this study, we investigated gene expression, neurotropic activity, and level of NGF and FGF-2 in cultured heart and brain ECs clones derived from myocardial and cerebral capillaries, respectively. Our key finding is that NGF is expressed by both cell types, while FGF-2 is expressed only in brain ECs. The OGD insult significantly decreased the levels of both growth factors indicating that an insult mimicking an ischemic event induces an opposite effect as compared to hypoxia, which is known to increase the release of both angiogenic (Calvani et al. 2006) and neurotropic (Kim et al. 2004) factors.

Previous studies have shown that NGF is produced by and exerts pro-survival effects on cardiac myocytes (Caporali et al. 2008) and is also produced by hematopoetic inflammatory cells at the site of injury, which are in part responsible for cardiac repair (Hasan et al. 2006; Frangogiannis 2008). In addition, we now propose that heart ECs represent another source of NGF. Our finding that OGD induced a decrease in NGF release by cultured heart ECs is in line with prior in

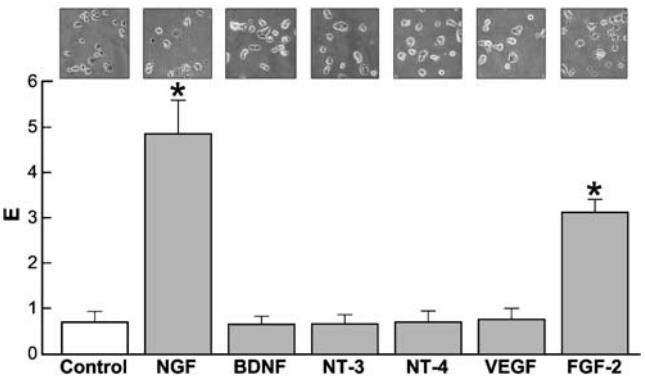

Figure 3. The specificity of PC12 bioassay towards different neurotrophins and angiogenic factors. PC12 cells were treated with 50 ng/ml of each individual growth factor (*gray bars*) or left untreated (control, *open bar*). After 2 d of treatment, the cultures were photographed and neuronal differentiation was evaluated by measuring elongation factor (*E*) as described under "Materials and Methods". Data are mean ± SD of three different experiments (n=12–18). *p<0.01 vs. control.

vivo studies that indicated lack of expression of NGF in the endothelial cells of capillaries at the site of the ischemic cardiac infarct, while its expression is increased in capillary pericytes (Hiltunen et al. 2001). OGD-induced NGF down-regulation in heart ECs (both in vitro and in vivo) may represent an adaptive response to sympathetic overactivity in heart failure (Kaye et al. 2000). Another explanation for our findings that ECs reduce the secretion of NGF as a result of an ischemia-like insult may be that in vivo a potential excesses of NGF could amplify the recruitment of TrkA-expressing inflammatory hematopoetic cells to the site of myocardial injury, thus exacerbating the damage. Therefore, we suggest that the levels of heart-derived NGF are strictly regulated to preserve cardiac homeostasis and to prevent arrhythmias due to sympathetic hyper-innervation following myocardial infarction (Hasan et al. 2006) and that heart-derived EC clones preserve this property upon propagation in culture. While we validated our findings using several distinct heart and brain microcapillary EC lines, further experiments are required to extend our findings to the heart and brain microvascular endothelium in vivo.

Endothelial cells are heterogeneous in terms of their structure depending on species, organ, type of blood vessel,

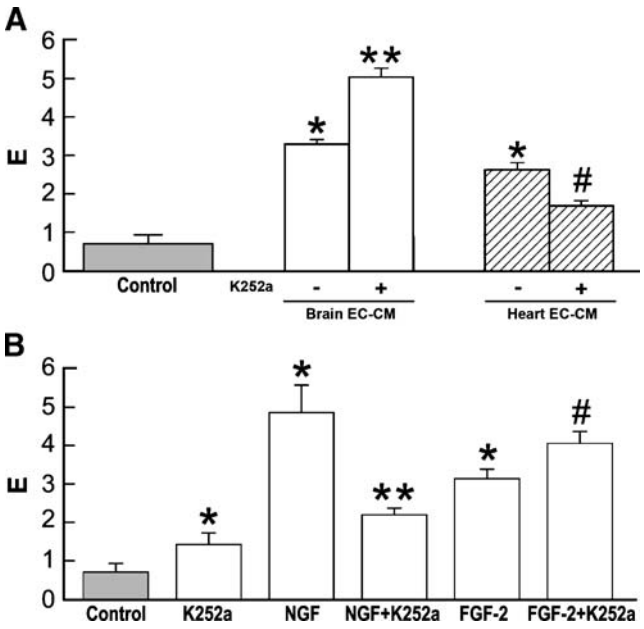

Figure 4. The inhibitory effect of K252a on neurotropic activity of brain and heart ECs CM and exogenous treatment with NGF or FGF-2 using the PC12 bioassay. *a* PC12 cells were treated with 50% heart (*stripped bars*) or brain (*open bars*) CM in the presence or absence of 100 nM K252a. Data are mean ± SD of four different experiments (n=10–15). *p<0.01 vs. control, **p<0.01 vs. control, #p<0.01 vs. CM 50%. *b* PC12 were treated with 50 ng/ml of NGF or FGF-2 in the presence or absence of 100 nM K252a. The control cells were treated with 0.1% DMSO or 100 nM K252a alone dissolved in 0.1% DMSO. Data are mean ± SD of three different experiments (n=9–12). *p<0.05 vs. control, **p<0.01 vs. NGF, #p<0.05 vs. FGF-2.

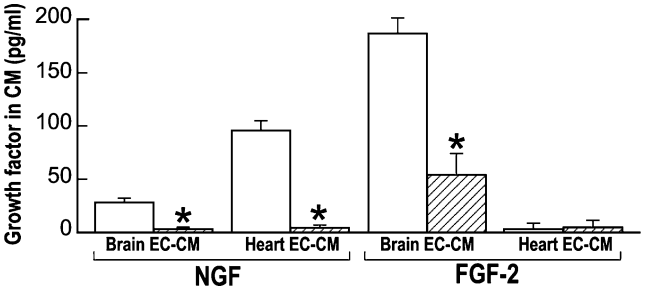

Figure 5. NGF and FGF-2 concentrations in the CM of heart and brain ECs under normoxia and OGD conditions measured by ELISA. Heart and brain ECs cultures were exposed to 6–8 h of OGD insults (*stripped bars*) or grown under normoxic conditions (*open bars*), the medium was collected, filtered, and submitted for growth factor-specific ELISAs. The level of growth factors is expressed as picogram per milliliter calculated from respective standard curves. Data are mean ± SD of three different experiments (n=5–7). *p<0.01 vs. normoxic conditions.

and even within individual vessel (Gumkowski et al. 1987; Pries and Kuebler 2006). Furthermore, ECs from divergent origin were shown to display marked differences in the extent of their responsiveness to growth factors stimulation (Harvey et al. 2002). This EC heterogeneity impacts hemodynamic conditions, such as oxygen transport, vascular control, coagulation, inflammation, and atherosclerosis (Forster et al. 2006; Pries and Kuebler 2006). The epigenetic hypothesis for EC heterogeneity assumes that the tissue-specific properties of ECs are governed by micro-environmental factors, such as cell–cell and cell–matrix interactions, partial pressure of oxygen, mechanical forces, and growth factors. The heart microcapillary ECs investigated in this study did not express and release FGF-2 as compared to brain microcapillary ECs. These findings are in line with previous reports indicating heterogeneity of FGF-2 expression and secretion by capillary ECs from the brain (Schweigerer et al. 1987), adrenal cortex (Lee et al. 2006), lung (Wedgwood et al. 2007), and circulating peripheral blood ECs (Shaked et al. 2005). The high level of expression of FGF-2 by brain ECs may be related to its role in the autocrine maintenance of brain capillary angiogenesis. Indeed, treatment of the brain ECs used in the present study with FGF-2-induced expression of pro-angiogenic genes and downregulation of anti-angiogenic genes (Yue et al. 2006). Therefore, we hypothesize that local physiological and pathological conditions differentially modulate synthesis and secretion of FGF-2 and NGF in heart and brain ECs. Based on our in vitro findings, we propose that the differential expression and secretion of NGF and FGF-2 in heart and brain ECs may differentially support the maintenance demand of the microcapillary microenvironment.

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