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Nerve Growth Factor-Induced Protection of Brain Capillary Endothelial Cells Exposed to Oxygen–Glucose Deprivation Involves Attenuation of Erk Phosphorylation

Shimon Lecht · Hadar Arien-Zakay · Cezary Marcinkiewicz · Peter I. Lelkes · Philip Lazarovici

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Abstract Nerve growth factor (NGF) was recently characterized as an angiogenic factor inducing proliferation, migration, and capillary sprouting in endothelial cells (ECs) of different vascular beds. While NGF neuroprotective effects on neurons were described, its survival-inducing effects on brain capillary ECs were not yet addressed. Using a model of oxygen–glucose deprivation (OGD) followed by reoxygenation, we demonstrated that NGF conferred protection in brain capillary ECs. These cells express TrkA and $p75^{NTR}$ receptors and respond to NGF by stimulation of Erk1/2 phosphorylation and stimulation of proliferation and migration. The NGF protective effect was dose-dependent, inhibited by NGF/TrkA antagonist, K252a, and required presence of NGF during both OGD and reoxygenation phases while the major protective effect was related to decreased cell death during the reoxygenation phase. A causal relationship was found between NGF-induced protection and attenuation of OGD-induced Erk1/2 phosphorylation, supporting the death-promoting role of insult-induced Erk1/2 phosphorylation in the brain capillary ECs. These results emphasize the importance of NGF in the process of EC survival in response to ischemic injury and suggest fine-tuning regulation of Erk1/2 phosphorylation, extending the neuroprotective impact of NGF from sympathetic neuroendocrine cells to brain capillary ECs as the other element in the neurovascular tandem.

Keywords Nerve growth factor · Erk1/2 · Oxygen–glucose deprivation and reoxygenation · Protection · Endothelial cells

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S. Lecht · H. Arien-Zakay · P. Lazarovici (✉)
Department of Pharmacology and Experimental Therapeutics,
School of Pharmacy, Faculty of Medicine,
The Hebrew University of Jerusalem,
POB 12065, Jerusalem 91120, Israel
e-mail: philipl@ekmd.huji.ac.il

C. Marcinkiewicz
Department of Biology, Temple University,
Philadelphia, PA, USA

P. I. Lelkes
Laboratory of Cellular Tissue Engineering,
School of Biomedical Engineering, Science and Health Systems,
Drexel University,
Philadelphia, PA, USA

Introduction

Over the last decade, some of the complex molecular mechanisms of neuronal cell death have been addressed (Kazantsev 2007) and many drugs with putative brain neuroprotective effects have been evaluated (Bordet et al. 2007). However, despite impressive progress, a clinically proven neuroprotective therapy, which would effectively ameliorate brain injury and neurodegeneration, still does not exist (Bordet et al. 2007). An emerging consensus in the field of neuroprotection research suggests that an approach based on using single agents for neuronal rescue may not suffice (Lo 2008). Central nervous system neurons must be considered as part of the "neurovascular unit" (Lo 2008), a concept which specifically emphasizes the significant role

of homeostatic interactions between the neurons and the endothelial cells (ECs) of brain capillaries. Therefore, protecting neurons alone may be not sufficient and future neuroprotective strategies and therapies ought to consider also brain capillary ECs. Indeed, recent studies of the crosstalk between the cardiovascular and the neuronal systems implicate an important role to neurotrophic and angiogenic factors in neuroprotection (Lazarovici et al. 2006; Zacchigna et al. 2008). Typical angiogenic factors such as vascular endothelial growth factor (VEGF; Yamakawa et al. 2003) and basic fibroblast growth factor (FGF-2; Calvani et al. 2006), and neurotrophins such as brain-derived neurotrophic factor (BDNF; Zacchigna et al. 2008) induced survival effects on ECs from different cardiovascular beds. Nerve growth factor (NGF) was previously shown to induce reparative angiogenesis in an ischemic tissue (Emanueli et al. 2002) and to prevent apoptosis of ECs in ischemic wounds of diabetic mice (Graiani et al. 2004; Salis et al. 2004). However, the protective effect of NGF on brain ECs is unknown and deserves investigation.

In previous studies, using PC12 sympathetic neuronal cultures and a home-made oxygen–glucose deprivation (OGD) device, we generated an in vitro model of neuronal ischemia mimicking the brain stroke ischemic/reperfusion injury. In this model, the cells were transiently exposed to OGD for different periods of time followed by a steady insult of 18-h reoxygenation (reperfusion). The neuroprotective effects of the anti-Parkinsonian drug, monoamine oxidase B inhibitor, rasagiline (Azilect™; Abu-Raya et al. 1999), the antioxidants carnosine and homocarnosine, the superoxide dismutase mimetic compound Tempol (Tabakman et al. 2002), and NGF (Tabakman et al. 2005) were characterized in this pharmacological model of ischemia.

Polyoma middle T-transformed mouse brain capillary ECs, bEnd.3 (Montesano et al. 1990), retain numerous functional markers of untransformed primary ECs: they express von Willebrand factor, VEGF receptors, E-selectin, platelet-endothelial adhesion molecule-1, vascular cell adhesion molecule-1, mucosal vascular addressin cell adhesion molecule-1, endothelial NO synthase, and endomucin. These cells can bind heparin, can internalize acetylated low density lipoprotein, and produce capillary-like tubes in three-dimensional cultures (Zhu et al. 2003). They also form gap junction and express multiple tight junction proteins (Brown et al. 2007), which are reduced under hypoxic conditions (Koto et al. 2007). These cells have frequently been used as a convenient biochemical model to investigate gene expression in cerebral microvascular ECs (Kallmann et al. 2002) and were also employed as a pharmacological model to elucidate the mechanism of cell death, hypoxic preconditioning, and protection upon hypoxia or OGD (Andjelkovic et al. 2003; Zhu et al. 2003; Kim et al. 2004; Lee et al. 2007). Since reoxygenation insult represents an important contributor in stroke and was not investigated in bEnd.3 model, we sought to develop and characterize the use of bEnd.3 ECs as an in vitro OGD/reoxygenation model. These two consecutive insults model will enable identification of signaling pathways involved in ischemia (Zhu et al. 2003) and to develop protective approaches for brain capillary ECs.

Despite advances in understanding the protective effects of classical angiogenic factors, VEGF (Chow et al. 2001) and FGF-2 (Yang and de Bono 1997), against EC death induced by different insults, little is known about the protective mechanisms of classical neurotrophins such as BDNF and NGF (Kim et al. 2004). Activation of Erk1/2 protects neurons from apoptosis (Xia et al. 1995) as well as being detrimental in ischemic brain and cortical neuron injury (Alessandrini et al. 1999). Increased Erk1/2 phosphorylation has been noted in the vulnerable penumbra after acute ischemic stroke in humans as well as in mouse models (Slevin et al. 2000).

In the present study, we characterized NGF ability to protect brain capillary ECs against an ischemic insult; the ischemic insult, in turn, was temporally related to the phosphorylation of Erk1/2, which was partially reversed by NGF. These findings support the role of NGF in the protection of brain capillary ECs in addition to that of VEGF and FGF-2, extending the neuroprotective impact of NGF from sympathetic neuroendocrine cells to brain capillary ECs as the other element in the neurovascular tandem.

Materials and Methods

Cell Culture

Mouse brain capillary ECs, bEnd.3, were obtained from ATCC (Manassas, VA, USA). bEnd.3 cells were cultured in 75-cm^2 tissue culture flasks (Nunc, Denmark) with growth medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 mg/ml glucose, 10% fetal calf serum (FCS), 2 mM L-glutamine, 10,000 U/ml penicillin, and 100 μg/ml streptomycin (Beit Haemek, Israel). PC12 pheochromocytoma cells were maintained in culture as previously described (Tabakman et al. 2005). 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 1:10 ratio after trypsinization of the cultures with 0.25% trypsin solution (Beit Haemek, Israel; Montesano et al. 1990). Twenty-four hours before the experiments, the cultures were washed three times with DMEM only and the medium was replaced with 1% FCS glucose-containing DMEM with 200 mM L-glutamine, 10,000 U/ml penicillin, and 100 μg/ml streptomycin. Visual evaluation of the cells

was performed using an inverted phase-contrast light microscope (Nikon® Eclipse TS-100).

Oxygen–Glucose Deprivation/Ischemic Protocol

To induce the OGD insult, bEnd.3 cells were cultured in 12-well plates for 48 h and introduced into an ischemia chamber (Fig. 1). Oxygen deprivation was achieved by replacing air/CO_2 mixture with a mixture of 5% CO_2/95% N_2 (Fig. 1a), which was humidified by passing the gas through a water-containing cylinder (Fig. 1b) to prevent endothelial cultures from drying out. After placing the culture plates into the ischemia chamber device (Fig. 1c) and hermetically closing the lid (Fig. 1d), the oxygen level in the device rapidly fell below 1% and stayed constant, as monitored on-line during the entire duration of an experiment using a digital oxygen analyzer (Fig. 1e, 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 (Fig. 1f). To generate glucose starvation, in addition to the hypoxic insult, the regular DMEM (4.5 mg/ml glucose) was aspirated immediately before initiation of OGD experiment, and the cultures were washed three times with glucose-free DMEM (ischemic medium) followed by addition of 1 ml of the same ischemic medium. Thereafter, the cultures were introduced into the ischemia chamber. The ischemic/reperfusion insult was carried out in two phases (Li et al. 2006; Yang et al. 2007): phase I, the cells were exposed to OGD for different periods of time (2–10 h); phase II, at the end of OGD phase, concentrated glucose solution was added to generate a final concentration of 4.5 mg/ml in the OGD-exposed cultures (in 1 ml final volume), and the plates were placed in a regular culturing incubator for 18 h under normoxic conditions, thus mimicking reoxygenation/reperfusion. This protocol preserves all the mediators accumulated in the media during phase I to further contribute to the insult during phase II. At the end of phase I or II, the medium was evaluated for lactate dehydrogenase (LDH) activity in the medium to estimate cell damage (death). Control cultures were maintained in the incubator with glucose-containing DMEM at normoxic conditions. The growth factors (NGF from Alomone Labs, Jerusalem, Israel; FGF-2 from Peprotech, Rocky Hill, NJ, USA) were added at different concentrations immediately before initiation of the OGD insult and were present during whole insult including the reoxygenation phase. The inhibitors (K252a from Fermentek, Jerusalem, Israel; PD98059 from Invitrogen, Carlsbad, CA, USA) were added to the cultures 1 h prior to addition of the growth factors and were present during phases I and II of the experiment.

LDH Release Cell Death Assay

Cell death was measured at the end of the OGD period and/or reoxygenation period by quantifying the activity of released LDH into the medium, using a commercial kit (Pointe, Canton, MI, USA). LDH activity was determined spectrophotometrically at 340 nm by following the rate of conversion of oxidized nicotinamide adenine dinucleotide (NAD^+) to the reduced form (NADH). Total LDH (extracellular+intracellular) was obtained by freezing and thawing the cultures. The basal LDH release measured in cultures maintained under normoxia was subtracted from all the experimental values. OGD/reoxygenation-induced cell death was expressed as percentage of released LDH from total LDH activity (Tabakman et al. 2002).

Measurements of Erk1/2 Phosphorylation by Western Blotting

In order to isolate total cellular protein, the cells were lysed on ice for 30 min using cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Thereafter, the lysed cells were centrifuged for 10 min at 14,000 rpm. Following

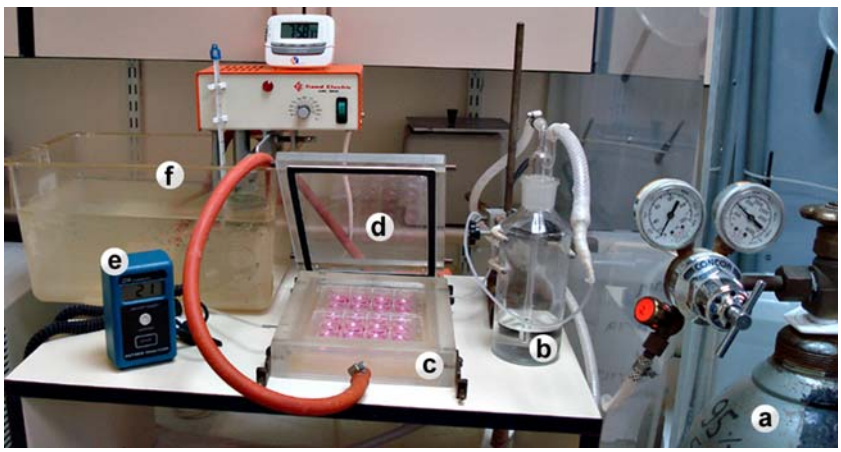

Figure 1 Setup for generating OGD in cultured cells. (*a*) 95% N_2/5% CO_2 gas tank with pressure regulator, (*b*) water trap, (*c*) hypoxia chamber that can accommodate two tissue culture plates, (*d*) the cover of the camber with rubber fittings to ensure proper sealing, (*e*) oxygen analyzer, (*f*) thermostated water bath

centrifugation, the supernatant was collected and the amount of total protein was quantified using the Bradford reagent assay (Bio-Rad, Hercules, CA, USA). For Western blotting, equal amounts of protein were loaded on 10% polyacrylamide gels, separated by SDS–PAGE (110 V for 1.5 h), and transferred on ice to nitrocellulose membranes (90 V for 1.5 h; Whatman, Germany). Non-specific binding was blocked by incubation of the membranes for 2 h at room temperature (RT) with 5% non-fat powdered milk in Tris-buffered saline containing 0.1% Tween-20. Immunodetection was performed using primary antibodies (1:1,000; Cell Signaling Technology) against phospho- or pan-Erk1/2. The antibodies were incubated overnight with the membranes at 4°C. Following incubation, the membranes were washed and then incubated for 1 h at RT with a horseradish peroxidase-conjugated goat anti-rabbit (1:10,000, Jackson ImmunoResearch, West Grove, PA, USA) secondary antibody; the blots were visualized using an ECL reagent (Pierce, Rockford, IL, USA). Thereafter, the same membranes first exposed to anti-phospho-Erk1/2 antibodies were washed, incubated for 30 min at room temperature in Restore Western Blot Stripping Buffer (Pierce), and then incubated with the anti-pan-Erk1/2 antibody. The developed films were scanned by a flatbed scanner and the densitometric analysis was performed with the Quantity One 1-D software, version 2003 (Bio-Rad). For each band, the densitometric values were obtained for the phospho-Erk1/2 and the pan-Erk1/2 of the same treatment. The background of each film was subtracted and the relative density of phospho-Erk1/2 was divided by the density of pan-Erk1/2. In order to compare between different treatments, the relative phospho-Erk1/2 phosphorylation was normalized to the values of untreated (control) cultures. The data are presented as fold of the normalized basal/control phosphorylation levels (Tabakman et al. 2004).

Cell Proliferation

After trypsinization and counting in a hemocytometer, 2×10^3 ECs were added to each well of 96-well tissue culture plates (Nunc, Denmark) and left to adhere overnight. Thereafter, EC cultures were supplemented with growth factors in the presence or absence of different inhibitors in 1% FCS glucose-containing DMEM with 2 mM L-glutamine, 10,000 U/ml penicillin, and 100 μg/ml streptomycin. Upon 3 days of treatment, cell numbers were estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay according to the manufacturer's instructions (Sigma, Israel). Briefly, MTT was added to the wells at a final concentration of 1 mg/ml for 40 min. At the end of the incubation period, the medium was aspirated and the cell monolayer was solubilized with DMSO. The optical density (OD) of the solution was measured at 570 nm using a FluorPlus spectrofluorometer (Tecan, Switzerland). The increase in cell number upon growth factor treatment was expressed as percentage increase in OD compared to untreated (control) cells.

Cell Migration

Endothelial cell migration was measured using a well-established wound-healing assay (Eccles et al. 2005). In brief, 1×10^5 cells/well were added to 12-well culture plates (Nunc) and left to adhere overnight. The next day, the medium was changed to 1% FCS glucose-containing DMEM with 2 mM L-glutamine, 10,000 U/ml penicillin, and 100 μg/ml streptomycin. Upon formation of a monolayer (after 2 days), migration experiments were initiated by scratching the EC monolayer with a small pipette tip, thus generating a cell-free area (wound) of ~1 mm width. The wounded cultures were washed three times with 1% FCS medium and pictures of the wounds at time 0 were taken. Thereafter, culture medium with different treatments was added and the cultures were allowed to migrate for 24 h. At the end of the migration experiment, another set of pictures was taken of the same spots. The area of wounds was measured using SigmaScan 5.0 program (Systat, Sweden). In order to assess cell migration at the wound edge and to calculate the area covered by migrating cells, the cell-free areas of the wounds at 24 h post-wounding was subtracted from the area of the wounds at 0 h time point and calculated as a percentage of untreated (control) cultures.

Analysis of Gene Expression by RT–PCR

Total RNA was isolated from cell cultures using the SV total RNA isolation kit (Promega, Madison, WI, USA) according to the protocol recommended by the manufacturer. 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. The following mouse-specific primer pairs were used: (1) TrkA sense—GGT ACC AGC TCT CCA ACA CTG AGG; antisense—CCA GA ACG TCC AGG TAA CTC GGT G; product size 198 base pairs (bp); (2) $p75^{NTR}$ sense—TGC AGT GTG CAG ATG TGC CTA TGG C; antisense—AGG AAT GAG CTT GTC GGT GGT GCC G; product size 447 bp; (3) 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 PCR reactions of TrkA and $p75^{NTR}$ 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 60°C, 2 min at 72°C each, 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 PCR reactions 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).

Confocal Immunofluorescence

For visualization of TrkA expression, bEnd.3 endothelial cells were plated on cover slides and left overnight to adhere. The following day, the adherent cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA), washed three times with PBS, blocked with 0.5% bovine serum albumin (BSA) for 1 h at 37°C, and incubated overnight at 4°C with rabbit polyclonal anti-TrkA 203 antibody, kindly provided by Dr. David Kaplan, University of Toronto, Canada. After washing with PBS, secondary antibody FITC-conjugated goat anti-rabbit IgG (1:500; Chemicon, Billerica, MA, USA) was applied for 40 min at RT. In the negative control experiments, the primary antibody was omitted while the other steps remained the same. The cells were examined in a FluoView FV300 confocal laser scanning microscope (Olympus, Japan; Arien-Zakay et al. 2009).

Statistics

The results, presented as the mean ± SD of at least three independent experiments (n=9–15), were evaluated using the InStat3 statistics program (GraphPad, La Jolla, CA, USA).

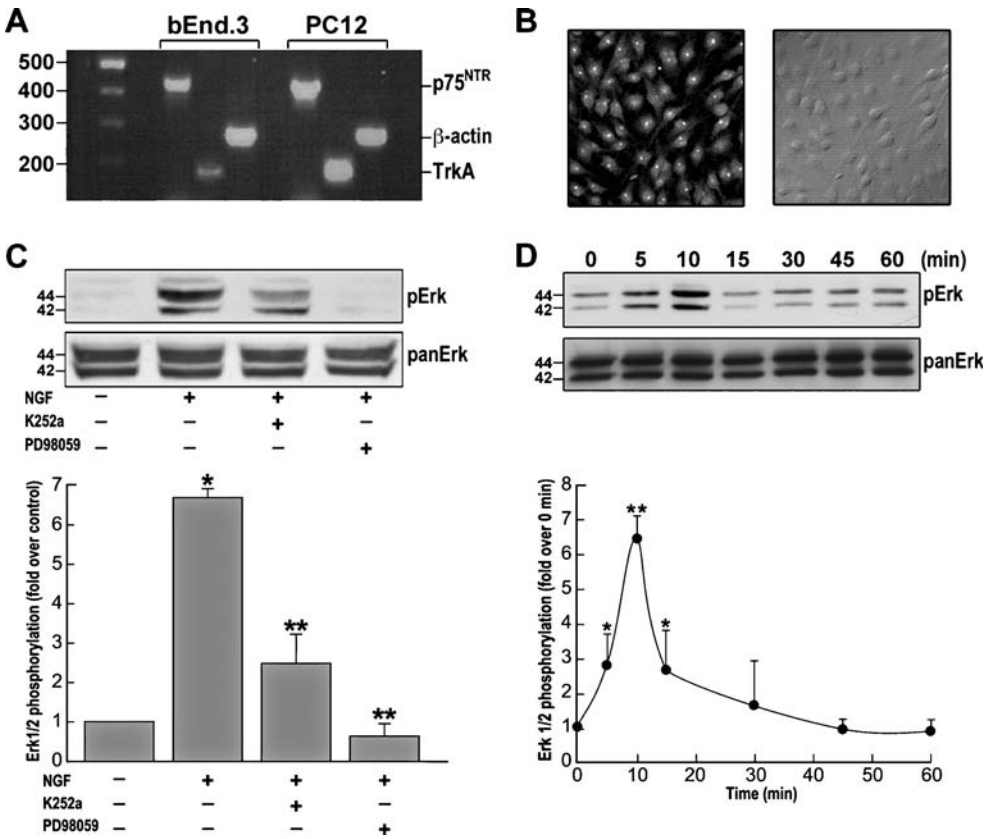

Figure 2 NGF receptors in brain capillary endothelial cells. **a** Evaluation by RT–PCR of NGF receptors $p75^{NTR}$ and TrkA in endothelial cells (*bEnd.3*), PC12 neuronal cells were used as positive control. *Numbers on the left* indicate DNA ladder size. **b** Confocal microscopy visualization of TrkA immunostaining in endothelial cells (*left image*) and phase-contrast image of the same filed (*right image*). **c** NGF-induced Erk1/2 phosphorylation is blocked by TrkA antagonist K252a (100 nM) and Erk1/2 inhibitor PD98059 (20 μM). *p<0.01 vs. untreated; **p<0.01 vs. NGF-treated. **d** Time course of NGF-induced activation of Erk1/2 phosphorylation in endothelial cells. The cultures were treated with 10 ng/ml NGF for the indicated time periods. The cell protein was extracted and the Erk1/2 phosphorylation was measured and calculated as described under the "Materials and Methods" section. *p<0.05; **p<0.01 vs. untreated. *Upper panels* in (**c**) and (**d**) present typical Western blots of Erk1/2 phosphorylation. The results presented as the mean ± SD of four independent experiments (n=12–15)

Table 1 NGF-induced brain capillary endothelial cells proliferation and migration are mediated by TrkA receptor and Erk1/2 phosphorylation

	Proliferation	Migration
Control	100%	100%
FGF-2	136%±13%*	116%±1%*
NGF	125±7%*	109±1%*
K252a	100±4%	100±2%
NGF+K252a	101±4%**	102±2%**
PD98059	100±2%	100±3%
NGF+PD98059	104±2%**	100±4%**

Serum-starved brain capillary endothelial cells were submitted for proliferation and migration experiments using MTT and wound-closure assays, respectively, as described under the "Materials and Methods" section. To initiate proliferation or migration of the cells the cultures were treated with 10 ng/ml NGF in the presence or absence of 10 nM TrkA antagonist K252a or 20 μM Erk1/2 inhibitor PD98059. FGF-2 (10 ng/ml) was used as a positive control. The results presented as the mean ± SD of four independent experiments (n=12–15)

*p<0.01 vs. control; **p<0.01 vs. NGF

Statistically significant differences between experimental groups were determined by analysis of variance with Bonferroni post hoc test with p<0.05 considered significant.

Results

NGF Receptors and Signaling in bEnd.3 Cells

Primary brain capillary ECs proliferate in response to NGF, express NGF receptors, and secrete NGF after inflammation (Kallmann et al. 2002; Moser et al. 2004). Similarly, cloned brain capillary ECs, bEnd.3, express BDNF receptors and secrete BDNF upon hypoxia, suggesting a role for BDNF in response to brain ischemia (Kim et al. 2004). However, expression of NGF and its receptors has not yet been investigated in bEnd.3 ECs. As seen in Fig. 2a, mRNA species for both NGF receptors, TrkA and $p75^{NTR}$, are expressed in these cells. The expression of TrkA receptor at the protein level was also validated by immunofluorescence (Fig. 2b). Furthermore, NGF rapidly (Fig. 2d) stimulated Erk1/2 phosphorylation (10 min) 6.5-fold over basal activity; this process was inhibited by 62±10% by the TrkA partial agonist, K252a, and completely blocked by the Erk1/2 inhibitor PD98059 (Fig. 2c). In order to further characterize bEnd.3 ECs, 10 ng/ml NGF stimulated 25±7% and 9±1% of their proliferation and migration, respectively; both of these processes were blocked by K252a and PD98059 (Table 1) as previously demonstrated for ECs from peripheral cardiovascular beds (Cantarella et al. 2002; Dolle et al. 2005; Rahbek et al. 2005), indicating that NGF effects on bEnd.3 ECs are mediated by TrkA-Erk1/2 signaling.

The Relationship Between OGD-Induced Cell Death and Erk1/2 Phosphorylation

A mouse brain capillary EC line, bEnd.3, was used as an in vitro OGD/reoxygenation model using a validated pharmacological paradigm previously employed and validated by us using neuronal PC12 cells (Tabakman et al. 2005). The ECs were first exposed to an OGD insult of different durations followed by a second phase "reoxygenation insult" during which the cells are cultured at ambient oxygen levels for another 18 h. Cell death was measured at different time points of exposure to OGD before and after 18 h of reoxygenation (Fig. 3). As seen in Fig. 3 under

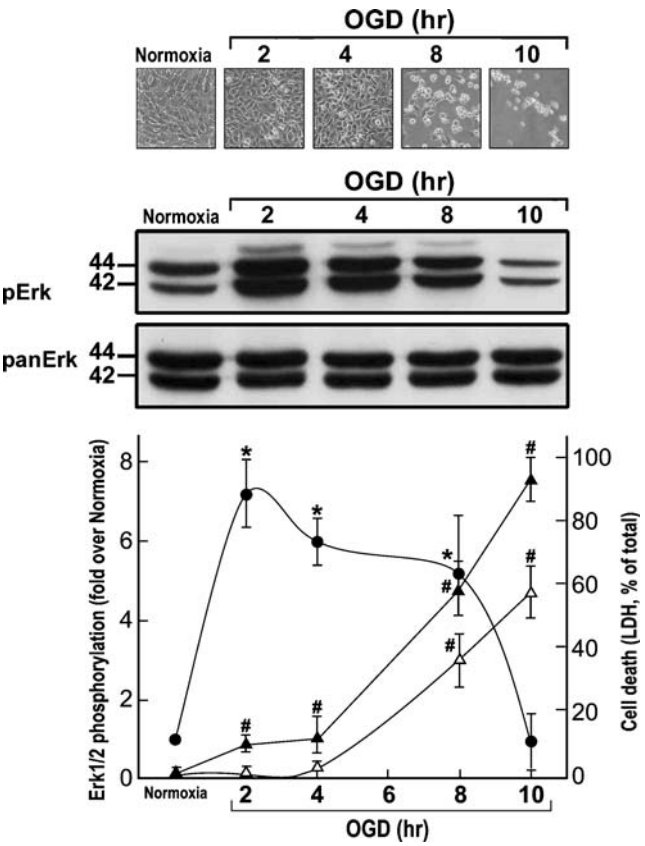

Figure 3 Time course of OGD/reoxygenation-induced brain capillary endothelial cell death and Erk1/2 phosphorylation. Cultures of 170,000 cells/well were exposed to OGD of different duration followed by a constant period of 18 h reoxygenation. LDH activity released to the medium was measured at the end of OGD insult (*white triangles*) and after OGD/reoxygenation (*black triangles*). Total protein from the cultures was extracted and 40 μg protein/sample was processed for Western blotting experiments using phospho- and pan-Erk1/2 selective antibodies, *black circles* Erk1/2 phosphorylation. Erk1/2 phosphorylation and LDH activities were calculated as described under the "Materials and Methods" section. *Upper panel* presents a typical Western blot of Erk1/2 phosphorylation and micrographs of the cultures exposed to the insults before the LDH measurements. The results presented as the mean ± SD of four independent experiments (n=12–15). *,#p<0.01 vs. normoxia

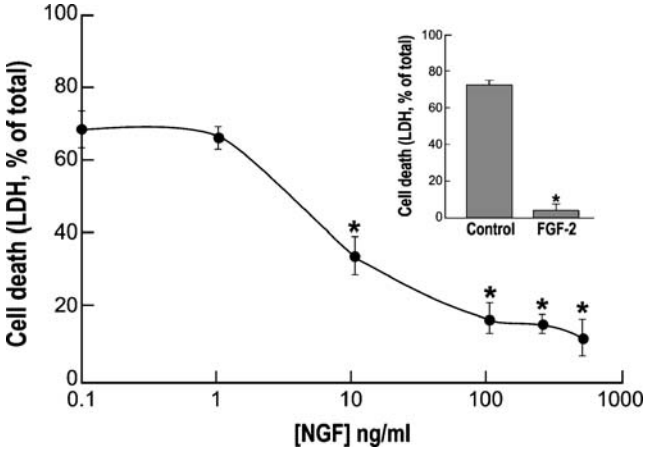

Figure 4 Dose response of NGF-conferred brain capillary endothelial cell protection. NGF treatment was initiated concomitantly with OGD insult that lasted for 8 h and cell death was measured at the end of reoxygenation phase. LDH activity was calculated as described under the "Materials and Methods" section. *Insert* FGF-2 (10 ng/ml) was used as positive control. The results presented as the mean ± SD of three independent experiments (n=9–12). *p<0.01 vs. control

normoxic conditions, EC death is minimal and less than 5± 2%. Increasing the duration of the OGD leads to an increase in cell death measured by LDH release as well as caspase-3 activity (data not shown). Exposure of the cells to the reoxygenation insult progressively increased EC death to a higher extent than the OGD insult alone. EC death in cultures exposed to 2 or 4 h of OGD followed by reoxygenation was most probably contributed by the reoxygenation phase. The cell death of cultures exposed for 8 or 10 h of OGD followed by reoxygenation was contributed by both OGD phase and reoxygenation phase insults. Light microscopic evaluation of the endothelial cultures clearly indicates destruction of the endothelial monolayer integrity before the reoxygenation phase due to cell detachment (Fig. 3, upper panel).

Since OGD increases Erk1/2 phosphorylation in neurons (Tabakman et al. 2004), we studied the time course of OGD-induced Erk1/2 activation in ECs. OGD insult induced a 5- to 7-fold increase in Erk1/2 phosphorylation during the first 8 h (Fig. 3).

NGF-Conferred Protection in Relation to OGD-Induced Erk1/2 Phosphorylation

To determine whether NGF confer protection against ischemic insults, the EC cultures were exposed to OGD/reoxygenation in the presence of different concentrations of NGF. As a positive control, we used FGF-2, a well-known angiogenic growth factor that increases endothelial resistance to oxidative stress (Yang and de Bono 1997). As seen in Fig. 4, the concentration of NGF providing half maximal protection (EC_{50}) against the combined OGD/reoxygenation insult was 6.5 ng/ml. Doses above 100 ng/ml provided maximal protection, which was similar to that provided by FGF-2 (Fig. 4—insert). To characterize the time window of NGF-induced protective effects, the cultures were treated with 10 ng/ml NGF under different protocols. As seen in Fig. 5a, the response of the ECs to exogenously added NGF is complex: exposure of the ECs in the presence of FGF-2, but not NGF, slightly protected by 11±2% the cell death of ECs at the end of 8 h OGD (phase I). However, upon application of a two-phase insult (8 h of OGD followed by 18 h reoxygenation), the presence of FGF-2 or NGF resulted in 65±3% and 34±4% reduction of cell death, respectively. Treatment with FGF-2 or NGF only during the reoxygena-

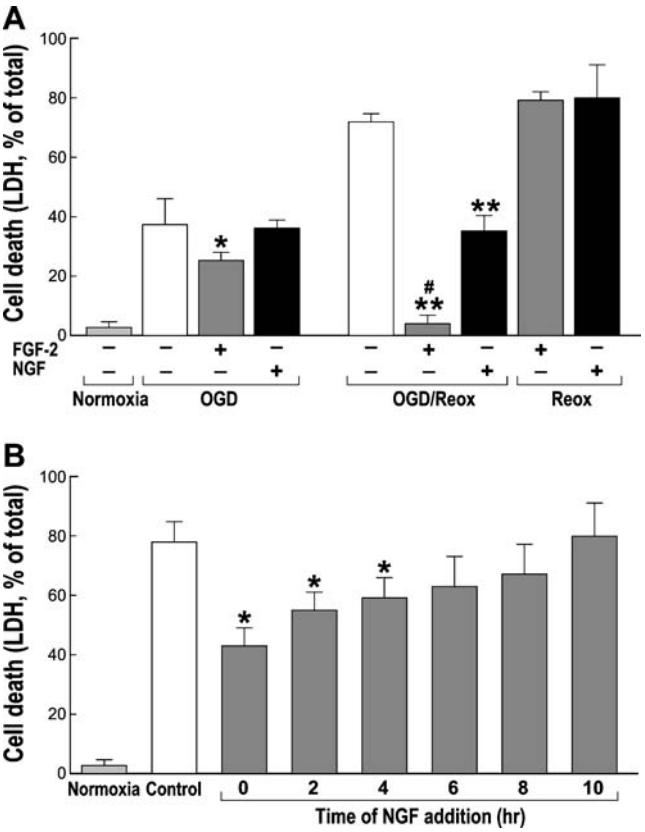

Figure 5 NGF-conferred protection of brain capillary endothelial cells towards OGD followed by reoxygenation insult. **a** NGF-induced protective effects require its continuous presence during the 8 h OGD and reoxygenation phases. NGF (10 ng/ml) was added concomitant with the insult and cell death was measured at the end of OGD or after reoxygenation (*OGD/Reox*). In an additional experiment, NGF (10 ng/ml) was added after OGD phase and cell death was measured at the end of reoxygenation phase (*Reox*). FGF-2 (10 ng/ml) was used as positive control. *p<0.05 vs. OGD untreated; **p<0.01 vs. OGD/Reox untreated; $^{\#}p$<0.05 vs. FGF-2 OGD. **b** Time course of NGF-induced protection during OGD insult up to 10 h duration. After OGD initiation, 10 ng/ml NGF was added concomitantly with the insult (0 h) or thereafter was added every 2 h of OGD insult to new insulted cultures, and cell death was estimated at the end of the reoxygenation phase. The results presented as the mean ± SD of three independent experiments (n=9–12). *p<0.05 vs. control

tion phase had no protective effects on EC cultures. These findings suggest that the NGF protective effect required its presence during both OGD and reoxygenation. In addition, the major NGF-induced protective effect is related to decreased contribution of reoxygenation-induced cell death. To elucidate the time frame required for NGF presence during the OGD phase to confer protection at the end of the reoxygenation insult, 10 ng/ml NGF was added at various time points after the initiation of the OGD and then maintained until the end of the reoxygenation phase. Figure 5b indicates that the addition of NGF during the first 0–4 h after the OGD insult induced a gradually reduced protection of the ECs during the reoxygenation phase, while there was no significant protection if NGF was added 6 h after the insult. Additional studies addressed the relationship between NGF-conferred protection and Erk1/2 phosphorylation. As shown in Fig. 6, OGD induced a 9.2±1.3-fold Erk1/2 phosphorylation and resulted in 72±2% cell death.

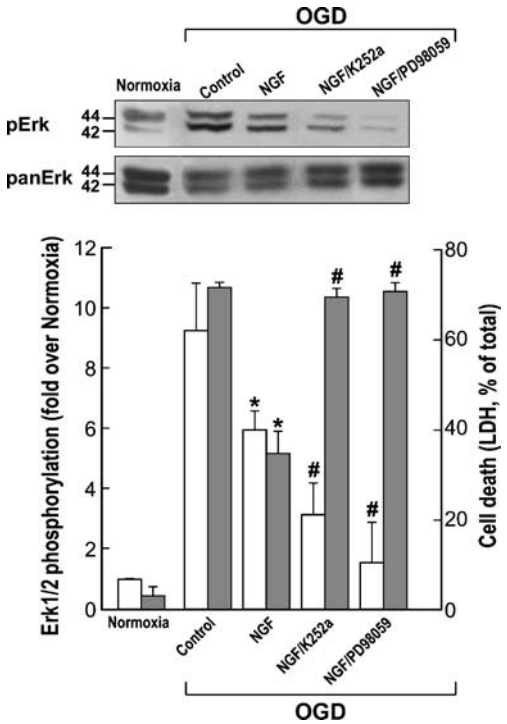

Figure 6 The relationship between OGD-induced Erk1/2 phosphorylation and brain capillary endothelial cells death upon treatment with NGF. Brain capillary endothelial cell cultures were exposed for 8 h of OGD in the presence of NGF (10 ng/ml), NGF and K252a (10 nM), NGF and PD98059 (20 μM), or left untreated (*Control*). Cultures under normoxic conditions served as internal control (*normoxia*). Erk1/2 phosphorylation was measured at the end of OGD phase (*white bars*) and cell death (LDH activity, *gray bars*) at the end of reoxygenation phase. Erk1/2 phosphorylation and LDH activities were calculated as described under the "Materials and Methods" section. *Upper panel* presents a typical Western blot of Erk1/2 phosphorylation. The results presented as the mean ± SD of three independent experiments (n=9–12). *p<0.01 vs. control; $^{\#}p$<0.01 vs. NGF

Treatment with 10 ng/ml NGF during the OGD/reoxygenation reduced the Erk1/2 phosphorylation and cell death by 36±6% and 34±7%, respectively. However, in the presence of 10 nM K252a or 20 μM PD98059, a reduction of NGF-induced Erk1/2 phosphorylation by 66±11% and 83±13%, respectively, was observed, while the protective effect of NGF was abrogated. Control cultures treated with 10 nM K252a or 20 μM PD98059 alone exhibited cell death similar to that of untreated cultures (data not shown). These results lend additional support to the concept of a bimodal role of Erk1/2 in the regulation of neuronal and endothelial cell survival and death: under certain circumstances, Erk1/2 phosphorylation may be involved in promoting cell death while in other situations Erk1/2 phosphorylation may be involved in the protective effects leading to cell survival (Tabakman et al. 2005; Zhuang and Schnellmann 2006).

Discussion

Recent studies have led to the concept that endothelial dysfunction plays a role in ischemic injury via its interactions with the neuronal and glial cells. In this study, we developed an in vitro ischemic/reperfusion injury model of brain capillary ECs to extend the protective ability of NGF from sympathetic neuroendocrine cells (PC12 cells; Tabakman et al. 2004) to ECs as another element in the neurovascular unit. The endothelial cultures were exposed after the OGD insult to a constant reoxygenation period of 18 h. This time point was found optimal for the expression of apoptotic pathways in endothelial cells (Wang et al. 2004). Indeed, in the present ischemic model, cell death can largely be attributed to the OGD insult and further exacerbated by reoxygenation (Fig. 3). In this experimental system, we demonstrated, for the first time, a protective role of NGF for brain capillary ECs during an ischemic insult. Our data suggest that the protective effects of NGF occur mainly during the reoxygenation phase but that effectiveness of cytoprotection depends on the presence of this neurotrophin during the OGD, in particular the first 4 h (Fig. 5b). Treatment of oxygen- and glucose-deprived cells with NGF at the beginning of the reoxygenation phase, which is more clinically relevant, failed to confer any protection. This finding suggests that the critical window for NGF-induced protection is related to the cells entering cellular death pathways during the initial phases of the OGD as previously found for neurons (Tabakman et al. 2004). NGF effects on diverse neuronal cells appear to depend upon its concentration, expression levels and ratio of its receptors (TrkA, $p75^{NTR}$), their kinetics of phosphorylation, and downstream signaling pathways (Kaplan and Miller 2000). Therefore, a similar complex responsiveness might also be the case for brain capillary ECs (Figs. 2, 5,

and 6). The NGF-induced protective effects on bEnd.3 ECs are in line with its neuroprotective effects on neuronal cells (Sofroniew et al. 2001; Lazarovici et al. 2006) and support the hypothesis that NGF, similar to VEGF and BDNF, is a growth factor with dual neurotrophic and angiogenic effects (Kim et al. 2004; Lazarovici et al. 2006). Further experiments are required to extend present NGF protective effects to brain microvascular primary EC preparations and in animal models of brain ischemic damage.

The involvement of the Erk1/2 signaling in OGD/reoxygenation and protection provided by NGF appears to be complex. Figure 3 illustrates the importance of Erk1/2 phosphorylation in the OGD/reoxygenation insult reminiscent of previous studies indicating hypoxia-induced Erk1/2 activation in bEnd.3 ECs (Zhu et al. 2003; Kim et al. 2004). Although Erk1/2 activation is considered as a survival-signaling event, prolonged (8 h) strong stimulation of Erk1/2 phosphorylation by OGD (Fig. 3) appears to be related to EC death, consistent with the concept of the paradox of dual survival/death-promoting role for Erks in neurons, glial, and epithelial cells (Zhuang and Schnellmann 2006). The protective effects of NGF were correlated with levels of about 5-fold stimulation of Erk1/2 phosphorylation (Fig. 6), while lower levels of Erk1/2 phosphorylation obtained during co-treatment with NGF and K252a or PD98059 failed to provide protection. In line with these results, treatment of hypoxia-exposed brain capillary ECs with BDNF (Kermani and Hempstead 2007) resulted with attenuation of Erk1/2 phosphorylation in conjunction with increased EC survival. Also, OGD-induced cell death in primary brain capillary ECs was correlated with increased Erk1/2 phosphorylation, which was decreased upon VEGF or U0126 (MEK inhibitor) treatment resulting in EC protection (Narasimhan et al. 2009). These results in the primary ECs are similar to the NGF-induced attenuation of Erk1/2 phosphorylation in the bEnd.3 endothelial cell clone. Moreover, a similar situation was reported in epithelial cells in which prolonged Erk1/2 activation was related to apoptosis (di Mari et al. 1999), whereas a transient activation of Erk1/2, as found in response to NGF stimulation (Fig. 2), protected the cells from death (Arany et al. 2004). These findings emphasize that both duration and level of Erk1/2 activation play an important role in brain capillary EC survival. Strong and prolonged Erk1/2 activation was reported in humans who had experienced ischemic brain stroke (Slevin et al. 2000), whereas a partial protective effect of Erk1/2 inhibition has been reported in animal models of ischemia/reperfusion (Zhuang and Schnellmann 2006). Furthermore, a potential contribution of other MAPK members such as p38 and JNK demonstrated in the OGD model of PC12 cells (Tabakman et al. 2004) might be also considered in the bEnd.3 OGD model, a possibility under investigation in our laboratory.

Present results indicating that, under specific circumstances, NGF protected brain capillary ECs against OGD/reperfusion injury, together with studies reporting BDNF-mediated protection of these ECs (Kermani and Hempstead 2007), suggest a key role for neurotrophic factors in therapeutic angiogenesis (Cristofaro and Emanueli 2009). Furthermore, the protective effects of these factors on both cerebral endothelial and neuronal components strongly support the notion that the crosstalk between these two physiological systems may constitute a fundamental process that confers protective mechanism (Sofroniew et al. 2001; Lazarovici et al. 2006). Targeting this trophic coupling between the endothelium and the neurons under pathologic conditions may provide new therapeutic opportunities.

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