Neurochemistry International 55 (2009) 610–618

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Selective regulation of growth factor expression in cultured cortical astrocytes by neuro-pathological toxins

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ARTICLE INFO

Article history:
Received 19 December 2008
Received in revised form 2 June 2009
Accepted 3 June 2009
Available online 12 June 2009

Keywords:
Astrocyte
Cortex
Growth factor
LPS
Neuroprotection

ABSTRACT

Astrocytes are integrated in the complex regulation of neurodegeneration and neuronal damage in the CNS. It is well-known that astroglia produces a plethora of growth factors which might be protective for neurons. Growth factors prevent neurons from cell death and promote proliferation and differentiation of precursor cells. Previous data suggest that astrocytes may respond to toxic stimuli by a selective mobilization of guarding molecules. In the present study, we have investigated the potency of different pathological stimuli such as lipopolysaccharides, tumor necrosis factor α, glutamate, and hydrogen peroxide to activate cultured cortical astroglia and stimulate growth factor expression. Astroglial cultures were exposed to the above factors for 24 h at non-toxic concentrations for astrocytes. Growth factor expression was analyzed by real-time PCR, oligo-microarray technique, and ELISA. Insulin-like growth factor-1 was selectively down-regulated by lipopolysaccharides and tumor necrosis factor α, bone morphogenetic protein 6 by all stimuli. In contrast, lipopolysaccharides, tumor necrosis factor α, and glutamate increased leukemia inhibitory factor. Fibroblast growth factor 2 was up-regulated by lipopolysaccharides and tumor necrosis factor α and down-regulated by hydrogen peroxide. Besides hydrogen peroxide, all other stimuli promoted vascular epithelial growth factor A mRNA and protein expression. It appears that lipopolysaccharides but not tumor necrosis factor α effects on vascular epithelial growth factor A depend on the classic NFκB pathway. Our data clearly demonstrate that astroglia actively responses to diverse pathological compounds by a selective expression pattern of growth factors. These findings make astrocytes likely candidates to participate in disease-specific characteristics of neuronal support or damage.

1. Introductory statement

Neuroglia comprising micro- and macroglia, have diverse functions in the mammalian CNS. Microglia is mainly implicated in the control of inflammatory processes, whereas macroglia, encompassing oligodendrocytes and astrocytes, fulfill a magnitude of functions that are essential for the establishment and maintenance of brain circuits. Besides playing an important metabolic role for neurons, astrocytes are also approved to be part of inflammatory mechanisms within the CNS (Aloisi et al., 2000; Dong and Benveniste, 2001; Farina et al., 2005; Kipp et al., 2008). Astrocytes participate in brain prostanoid metabolism, thus modulating brain-intrinsic physiological processes in a paracrine manner through the synthesis of prostaglandins, thromboxanes, and leukotrienes (Kipp et al., 2008). This type of glia cell also synthesizes and secretes inflammation-related cytokines and chemokines under pathological conditions (Farina et al., 2007). The latter function may explain the importance of astroglial cells for inflammatory and degenerative processes in the brain (Darlington, 2005). Specific attention has been paid to astrocyte dysfunctions in various pathological disorders, among them multiple sclerosis (Williams et al., 2007), Parkinson's disease (Hald and Lotharius, 2005), amyotrophic lateral sclerosis (ALS) (Moisse and Strong, 2006), and Alzheimer's disease (Ting et al., 2007).

Besides their role in placing harmful pro-inflammatory molecules which promote neurodegeneration, astrocytes are concurrently important players for regenerative or protective processes by releasing growth factors which support neuronal survival and stability (Erkanli et al., 2007; Salmaso and Woodside, 2008; Tripathi and McTigue, 2008). It is evident that growth factor

Abbreviations: ALS, amyotrophic lateral sclerosis; BMP-6, bone morphogenetic protein 6; CNS, central nervous system; FGF-2, fibroblast growth factor 2; Glu, glutamate; H_2O_2, hydrogen peroxide; Iba-1, ionized calcium-binding adaptor molecule 1; IGF-1, insulin-like growth factor 1; IL, interleukin; LDH, lactate dehydrogenase; LIF, leukemia inhibitor factor; LPS, lipopolysaccharides; MA, metabolic activity; MT-3, metallothionein 3; NeuN, neuronal nuclei; qRT-PCR, quantitative real-time PCR; SPP-1, phosphoprotein 1; TGFβ-3, transforming growth factor beta 3; TNF-α, tumor necrosis factor α; VEGF-A, vascular epithelial growth factor A.

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doi:10.1016/j.neuint.2009.06.004

expression and availability is a well-described phenomenon in different pathological disorders such as epilepsy (Erkanli et al., 2007), multiple sclerosis (Chesik et al., 2007), stroke (Onori et al., 2009), and Parkinson's disease (Peterson and Nutt, 2008). In addition, distinct growth factors exhibit the potential to induce neuronal stem cell proliferation and differentiation (Caroni and Becker, 1992; Sailer et al., 2005; Hampton et al., 2007). Although the latter characteristic is thought to have a higher impact during embryogenesis (Hsu et al., 2007), such an option may also appeal after neuronal damage in the brain.

The purpose of the present study was to investigate whether astrocytes *in vitro* respond differently to the application of neurotoxins with respect to their capacity of growth factor expression. The following factors were chosen, since they mimic a variety of pathological events in the CNS: Lipopolysacharides (LPS) are symptomatically used to provoke sepsis-induced neuronal damage (Henry et al., 2008) or bacteria-induced inflammatory processes (Palsson-McDermott and O'Neill, 2004; O'Reilly et al., 2007), the cytokine tumor necrosis factor α (TNF-α) is implicated in different brain pathologies (Huang et al., 2005; Gosselin and Rivest, 2007), hydrogen peroxide (H_2O_2) is supposed to mimic oxidative stress (Watt et al., 2004), and glutamate (Glu) is noted for its excitotoxic effects in the brain and its relation to neurological disorders (Werner et al., 2000; Pitt et al., 2003).

2. Experimental procedures

2.1. Cell culture and treatment

Primary astrocytes were prepared from 2-to-4-day-old Balb/c mice (Harlan Winkelmann, Germany) as described previously (Kipp et al., 2007, 2008). Briefly, cerebral cortices were dissected, meninges removed and dispersed in Dulbecco's phosphate-buffered saline containing 1% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA), filtered through a 50-μm nylon mesh, and centrifuged at 400 × *g* for 5 min. Cells were re-suspended in Dulbecco's modified Eagle's medium (Invitrogen, Germany) containing 20% heat-inactivated fetal calf serum (PAA, Austria), streptomycin (0.5%) and fungizone (0.1%, both from Invitrogen, Germany). Finally, cells were seeded in 100 mm plates coated with poly-ornithine (Sigma, Germany) at a density of 1–2 × 10^6 cells/cm^2. Astrocytes were grown at 37 °C in a humidified incubator with 5% CO_2. Medium was replaced every third day. Upon reaching confluency, cells were trypsinized and replated at a lower density. This procedure was repeated twice. The resulting final astroglia culture is characterized by >95% homogeneity of glial fibrillary acidic protein positive cells and no microglial (Iba-1^+) or neuronal ($NeuN^+$) contamination (Pawlak et al., 2005). Contaminating cells were vimentin positive indicating that these cells are either fibroblasts or $GFAP^-$/$vimentin^+$ astrocytes (Goursaud et al., 2009).

For primary neuronal cell cultures, tissues were taken from Balb/c mice embryos at gestational day 15/16 and were cultured as described previously (Kajta et al., 2006). Briefly, pregnant females were anesthetized with CO_2 vapor, killed by cervical dislocation, and subjected to caesarean section. Cerebral cortices were dissected from fetuses. Tissues were minced into small pieces, then digested with trypsin (0.1% for 15 min at room temperature, Sigma, Germany), triturated in the presence of 10% fetal bovine serum (Gibco, Germany) and DNAse I (170 U per ml, Sigma, Germany), and finally centrifuged for 5 min at 100 × *g*. Cell pellets were suspended in neurobasal medium (Gibco, Germany) supplemented with B27 (0.2%, Gibco, Germany) an plated at a density of 1.5 × 10^5 cells per cm^2 onto poly-ornithine (Sigma, Germany)-coated 24-well plates (Greiner, Germany). This procedure typically yields cultures that contain >90% neurons and <10% supporting cells (mainly $GFAP^+$ astrocytes). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 8 days prior to viability analysis.

For gene expression and cell viability analysis, cells were treated for the indicated period with TNFα (Invitrogen, Germany), LPS (Invitrogen, Germany), Glu (Sigma, Germany) or H_2O_2 (Roth, Germany) in the given concentration. SN-50 NFκB inhibitor (50 μg/ml, Biomol, Germany, solved in water), and curcurmin (5 μM, Biomol, Germany, solved in DMSO) were applied 1 h prior cell stimulation. Proper controls were conducted to exclude that inhibitor or solvent application alone affects growth factor expression in primary astrocyte cultures.

2.2. Growth factor oligo GEArray

High-quality total RNA from astrocyte cultures for gene expression profiling experiments was obtained using ArrayGrade™ Total RNA Isolation kit (SABioscience, USA) according to the manufacturer's protocol. Prior to labelling RNA, quality and purity of RNA was tested by UV spectrophotometry in RNase-free 10 mM Tris-buffer (pH 8.0) and by RNA gel electrophoresis. UV spectrophotometry revealed that the extinction ratio 260:280 was >2.0 and 260:230 was >1.7. RNA was reverse transcribed and biotin-labelling performed using the TrueLabeling-AMP 2.0 kit (SABioscience, USA) according to the manufacturer's protocol. Biotinylated-UTP (10 mM, Roche Applied Science, Germany) and ultra pure water (Invitrogen, Germany) was used for RNA labelling. The obtained cRNAs were further purified using the ArrayGrade™ cRNA Cleanup kit (SABiosciences, USA). For gene expression profiling analysis, we used the Oligo Mouse Growth Factors GEArray System (SABioscience, USA) according to the manufacturer's protocol. This microarray analyzes the expression of 113 common growth factors which play a vital role under normal biological and pathological processes. Hybridization was performed over night at 60 °C. Detection of signal intensity was achieved using a Chemoluminescent Detection kit provided by the manufacturer, data extraction and analysis with a GEArray expression analysis system (SABiosciences, USA). Optical density of genes of interests is normalized against internal controls (three different house keeping genes).

Table 1
Primer sequences used far real-time PCR analysis.

	Sense	Antisense	AT
HPRT	gct ggt gaa aaggac ctc t	cac agg act aga aca cct gc	60 °C
IGF-1	cta cca aaa tga ccg cac ct	cac gaa ctg aag agc atc ca	60 °C
FGF-2	cca acc ggt acc ttg cta tg	tat ggc ctt ctg tcc agg tc	62 °C
VEGF-A	cca cgt cag aga gca aca tca	tca ttc tct cta tgt gct ggc ttt	60 °C
LIF	gcc tcc ctg acc atc acc at	gac ggc aaa gca cat tgc tg	57 °C
BMP-6	caa cgc cct gtc caa tga c	act ctt gcg gtt caa gga gtg	59 °C

For abbreviation see text.

2.2.1. Quantitative real-time PCR (qRT-PCR)

Gene expression was measured using the qRT-PCR technology (BioRad, Germany), QTM SYBR Green supermix (BioRad, Germany) and a standardized protocol as described previously (Kipp et al., 2008). Isolation of total RNA was performed with NucleoSpin kit (Macherey-Nagel, Germany). RNA concentration and purity were assessed using OD260 and OD260/OD280 ratio, respectively, and reverse transcribed using an Invitrogen M-MLV RT-kit and random hexanucleotide primers. Reactions were carried out in a mixture consisting of 2 μl cDNA, 2 μl RNAse-free water, 5 μl QTM SYBR Green supermix, and 0.5 μl primers (100 pmol/μl). Reactions were conducted in standard tubes using the MyIQ qRT-PCR detection system (BioRad, Germany) under following conditions: 10 min enzyme activation at 95 °C, 45 cycles of 15 s denaturation at 95 °C, 30 s annealing at individual temperatures, 30 s amplification at 72 °C, and 5 s fluorescence measurement at 72 °C. Primer sequences are shown in Table 1. Relative quantification was performed using the ΔCt method which results in ratios between target genes and a housekeeping reference gene (HPRT) (Kipp et al., 2008). As the validity of this method critically depends on the constant expression of the housekeeping gene, constant expression of HPRT was tested against other housekeeping genes (not shown). In each run, internal standard curves were generated by several fold dilutions of target genes. The concentration of the target genes was calculated by comparing Ct values in each sample with Ct values of the internal standard curve. Finally, data were expressed as the ratio of the amount of each transcript vs. the concentration of HPRT. Melting curves and gel electrophoresis of the PCR products were routinely performed to determine the specificity of the PCR reaction.

2.3. Cell viability assay

Lactate dehydrogenase (LDH) release was determined using the CytoTox 96® non-radioactive cytotoxicity assay (Promega, Germany) according to the manufacturer's instructions. Three vials per experiment were treated 1 h with a lysis solution containing Triton X-100 to obtain maximum LDH release. Results are given as percentage LDH release related to maximum LDH release. Metabolic activity was determined using the CellTiter-Blue® cell viability assay (Promega, Germany). Treatment with lysis solution served as negative control. The average of fluorescence intensity of Triton X-100-treated cells was subtracted from fluorescence values obtained from cultured cells. Interference of Triton X-100 with fluorescence signals was excluded. Data are given as percentages of control.

2.4. Western blot analysis

Nuclear protein was isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, USA) following the manufacturer's instructions. Cells were harvested by scraping, washed and pelleted by centrifugation. All subsequent manipulations were performed on ice. 1 mM phenylmethylsulfonyl fluoride, 1 μl/ml aprotinin, and 1 μl/ml leupeptin were added to the cell lysis solution to inhibit protein digestion. Nuclear proteins were separated on an 8% polyacrylamide gel electrophoretically and then transferred onto nitrocellulose membranes (BioRad) overnight. The nitrocellulose membranes after washing in PBS for 15 min were blocked with 5% fat-free milk solution in PBS for 15 min. The membranes were then incubated with anti-NFκB p65 (C22B4 antibody) or anti-Lamin A/C antibodies (both 1:1000, Cell Signaling, USA) diluted in 5% milk in PBS-Tween over night at 4 °C. After