Pharmacological Research 87 (2014) 80–86

Contents lists available at ScienceDirect

Pharmacological Research

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

Critical role of large-conductance calcium- and voltage-activated potassium channels in leptin-induced neuroprotection of N-methyl-D-aspartate-exposed cortical neurons

Maria Mancini[a,b,c], Maria Virginia Soldovieri[a], Guido Gessner[c], Bianka Wissuwa[d], Vincenzo Barrese[e], Francesca Boscia[e], Agnese Secondo[e], Francesco Miceli[e], Cristina Franco[e], Paolo Ambrosino[a], Lorella Maria Teresa Canzoniero[b], Michael Bauer[d], Toshinori Hoshi[f], Stefan H. Heinemann[c], Maurizio Taglialatela[a,e,*]

[a] Department of Medicine and Health Science, University of Molise, Campobasso, Italy
[b] Department of Science and Technology, University of Sannio, Benevento, Italy
[c] Center for Molecular Biomedicine, Department of Biophysics, Friedrich Schiller University and Jena University Hospital, Jena, Germany
[d] Center for Sepsis Control and Care, Jena University Hospital, Jena, Germany
[e] Department of Neuroscience, University of Naples "Federico II", Naples, Italy
[f] Department of Physiology, University of Pennsylvania, Philadelphia, USA

ARTICLE INFO

Article history:
Received 21 May 2014
Received in revised form 17 June 2014
Accepted 18 June 2014
Available online 26 June 2014

Keywords:
Calcium- and voltage-activated potassium channels (BK channels)
Leptin
N-methyl-D-aspartate (NMDA)
Neuroprotection
Paxilline
Iberiotoxin
Cortical neurons
Intracellular Ca^{2+} concentration

ABSTRACT

In the present study, the neuroprotective effects of the adipokine leptin, and the molecular mechanism involved, have been studied in rat and mice cortical neurons exposed to N-methyl-D-aspartate (NMDA) *in vitro*. In rat cortical neurons, leptin elicited neuroprotective effects against NMDA-induced cell death, which were concentration-dependent (10–100 ng/ml) and largest when the adipokine was preincubated for 2 h before the neurotoxic stimulus. In both rat and mouse cortical neurons, leptin-induced neuroprotection was fully antagonized by paxilline (Pax, 0.01–1 μM) and iberiotoxin (Ibtx, 1–100 nM), with EC_{50}s of 38 ± 10 nM and 5 ± 2 nM for Pax and Ibtx, respectively, close to those reported for Pax- and Ibtx-induced Ca^{2+}- and voltage-activated K^+ channels (Slo1 BK channels) blockade; the BK channel opener NS1619 (1–30 μM) induced a concentration-dependent protection against NMDA-induced excitotoxicity. Moreover, cortical neurons from mice lacking one or both alleles coding for Slo1 BK channel pore-forming subunits were insensitive to leptin-induced neuroprotection. Finally, leptin exposure dose-dependently (10–100 ng/ml) increased intracellular Ca^{2+} levels in rat cortical neurons. In conclusion, our results suggest that Slo1 BK channel activation following increases in intracellular Ca^{2+} levels is a critical step for leptin-induced neuroprotection in NMDA-exposed cortical neurons *in vitro*, thus highlighting leptin-based intervention via BK channel activation as a potential strategy to counteract neurodegenerative diseases.

Abbreviations: NMDA, N-methyl-D-aspartate; BK channels, large-conductance Ca^{2+}- and voltage-activated K^+ channels; Pax, paxilline; Ibtx, iberiotoxin; OGD, oxygen–glucose deprivation; K_{ATP}, ATP-sensitive K^+ channels; E, embryonic age; HBSS, Hank's Balanced Salt Solution; MEM, Minimum Essential Media; FBS, fetal bovine serum; HS, horse serum; DIV, days *in vitro*; HCSS, HEPES control salt solution; MS, media stock; HEK293T cells, human embryonic kidney 293T cells; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; Fura2-AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(21-amino-51-methylphenoxy)-ethane-N,N,N1,N1-tetraacetic acid penta-acetoxymethyl ester; EC_{50}, effective concentration 50; $[Ca^{2+}]_i$, intracellular calcium concentration; PI3K, phosphatidylinositol 3-kinase.

* Corresponding author at: Department of Medicine and Health Sciences, University of Molise, Via De Sanctis, 86100 Campobasso, Italy. Tel.: +39 0874 404851; fax: +39 0874 404778.
E-mail addresses: m.taglialatela@unimol.it, mtaglial@unina.it (M. Taglialatela).

http://dx.doi.org/10.1016/j.phrs.2014.06.010
1043-6618/

Introduction

Leptin, a 16-kDa peptide hormone produced by white adipocytes, controls appetitive behaviors by acting on hypothalamic neurons involved in food intake and energy expenditure [1]. Leptin receptors are expressed in diverse brain regions such as the hippocampus, the cortex, and the cerebellum, being often located at axonal and synaptic subcellular sites [2]. Extrahypothalamic actions of leptin are increasingly being recognized; for example, leptin influences synaptic plasticity in hippocampal neurons [3,4] and inhibits epileptiform-like activity in hippocampal [5–7] as well as in neocortical neurons [8]. Several studies have also highlighted the ability of leptin to exert both *in vitro* and *in vivo* neuroprotective effects against oxygen–glucose deprivation, hypoxia, ischemia, neurotrophic factor withdrawal, and excitotoxic or oxidative stimuli in neuronal populations from distinct brain areas [9–15].

Among the molecular mechanisms responsible for the effects of leptin, neuronal silencing via activation of potassium (K^+) channels appears to play a major role. Among leptin-sensitive K^+ channels, activation of ATP-sensitive K^+ channels (K_{ATP}) has been proposed to mediate leptin-induced suppression of excitability of hypothalamic neurons [16,17]. Similarly, leptin-induced insulin release suppression from pancreatic β cells also depends on K_{ATP} activation [18]. More recently, pharmacological evidence has suggested that large-conductance, Ca^{2+}- and voltage-activated K^+ channels (Slo1 BK channels), which are particularly abundant in axons and nerve terminals [19,20], where they stabilize the neuronal membrane potential and regulate excitatory neurotransmitter release [21–26], mediate at least part of leptin's effects of neuronal excitability. In fact, activation of BK channels mediates leptin-induced inhibition of gastric mucosal vagal afferents [27], and hippocampal neuronal firing [5] and epileptiform-like events [6]. Notably, BK channel activation exerts strong neuroprotective effects in animal models of cerebral ischemia [28], and attenuated cerebral edema and neurologic motor impairment after traumatic brain injury [29]. Activation of BK channels also seems to mediate leptin effects on primary hippocampal neuronal excitability during hypoxia [15].

Despite these results, direct evidence for BK channel activation by this adipokine during neuroprotection is lacking. Therefore, in the present study, the neuroprotective potential of BK channel activation by leptin and the underlying molecular mechanism(s) have been assessed in cortical neurons exposed to the ionotropic glutamate receptor agonist N-methyl-D-aspartate (NMDA), a classical excitotoxic insult. The results obtained indicate that leptin is endowed with significant neuroprotective effects in both rat and mouse cortical neurons exposed to NMDA; the pharmacological blockade of BK channels, or the lack of one (*Slo*1$^{+/-}$ mice) or both (*Slo*1$^{-/-}$ mice) *Slo*1 alleles fully counteracted leptin-mediated neuroprotection. Furthermore, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) monitoring in single mouse cortical neurons revealed that leptin (10–100 ng/ml) application prompted an oscillatory behavior in $[Ca^{2+}]_i$. These results reveal that the activation of BK channels is an obligatory step for leptin-induced neuroprotection, highlighting leptin-based intervention via BK channel activation as a potential strategy to treat neurodegenerative diseases.

Materials and methods

Animals

Animals were kept under standard conditions of temperature, humidity and light, and were supplied with standard food and water *ad libitum*. Animals were handled in accordance with the recommendations of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and in accordance with a protocol approved by the institutional animal care committees. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Pregnant Wistar rats were purchased from a commercial source (Charles River, Calco, Italy), while wild-type, heterozygous and *Slo*1 knockout FVB/NJ mice were obtained from Prof. R. Aldrich (University of Texas, Austin, TX, USA), and genotyped as previous described [30]. Briefly, genomic DNA from tail snips was amplified by PCR using primers Neo 5′ (5′-ATA GCC TGA AGA ACG AGA TCA GC-3′) and RA 14025 3′ (5′-CCT CAA GAA GGG GAC TCT AAA C-3′), amplifying the *Slo*1$^{-/-}$ allele product of 800 bp, and the exon 1 5′–3 (5′-TTC ATC ATC TTG CTC TGG CGG ACG-3′) and WT 3′–2 (5′-CCA TAG TCA CCA ATA GCC C-3′) amplifying the wild-type product of 332 bp.

Rat and mouse cortical cell cultures

Primary cultures of rat and mouse cortical neurons were prepared from embryos at 15–17 days of gestation; embryonic age (E) was calculated by considering E0.5 the day when a vaginal plug was detected. Briefly, pregnant animals were anesthetized with diethyl ether (Carl Roth GmbH & Co KG, Karlsruhe, Germany) and sacrificed by cervical dislocation. Cortical tissues from embryos were dissected in ice-cold medium (HBSS, Hank's Balanced Salt Solution, supplemented with 27 mM glucose, 20 mM sucrose, 4 mM sodium bicarbonate), centrifuged, and the resulting pellet was mechanically dissociated with a glass pipette. Cells were resuspended in plating medium consisting of Eagle's MEM (MEM, Earle's salts, supplied bicarbonate-free) supplemented with 5% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), 5% horse serum (HS, Sigma Aldrich, Taufkirchen, Germany), 2 mM L-glutamine, 20 mM glucose, 26 mM bicarbonate, and plated on 24-well plates (Thermo Fisher Scientific, Waltham, MA, USA) or on 18 mm glass coverslips (Glaswarenfabrik Karl Hecht KG, Sondheim, Germany) coated with 100 μg/ml poly(D)-lysine (Sigma–Aldrich) at a density of four embryo cerebral hemispheres/10 ml; when single-embryo dissections were necessary, resuspension volumes were changed accordingly. Glial replication was inhibited by 24 h exposure to 10 μM cytosine arabinofuranoside (Sigma–Aldrich) after 4 days *in vitro* (DIV). After this treatment, the medium was supplemented with 10% HS and partially substituted twice a week. All the experiments were performed at 12–16 DIV. HBSS, Eagle's MEM, and glutamine were purchased from Life Technologies (Oslo, Norway). All other reagents were from Sigma–Aldrich.

Cellular treatments and assessment of neuronal survival

Prior to drug exposure, cortical neurons were washed thoroughly to remove serum using HEPES control salt solution (HCSS, 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 20 mM HEPES, 15 mM glucose, 1.8 mM $CaCl_2$, 10 mM NaOH, pH 7.4). NMDA (Sigma–Aldrich) exposure was carried out in HCSS for 15 min at room temperature, followed by NMDA washout in media stock (MS, MEM supplemented with 20 mM glucose and 26 mM bicarbonate). Recombinant human leptin (R&D Systems Inc., Minneapolis, MN, USA; stock solution was 1 mg/ml dissolved in 20 mM Tris–HCl, pH 8.0) was used for mouse and rat cortical neurons, as similar affinities have been measured for human leptin at human, mouse, and rat leptin receptors [31]. Leptin was added simultaneously to NMDA exposure, as well as 15 min, 2 h, or 6 h before NMDA exposure, according to the experimental protocol. Paxilline, iberiotoxin, or NS1619 were added at the desired concentrations from stock solutions in DMSO (maximal final DMSO concentration was <1%) 15 min before leptin application and kept throughout the experiment. After NMDA exposure, cultures were washed several times with HCSS buffer and maintained in MS for 24 h.