European Journal of Pharmacology 674 (2012) 104–111

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

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

Molecular and Cellular Pharmacology

The noble gas argon modifies extracellular signal-regulated kinase 1/2 signaling in neurons and glial cells

Astrid V. Fahlenkamp [a,b,1], Rolf Rossaint [b], Hajo Haase [c], Hussam Al Kassam [b], Yu-Mi Ryang [d], Cordian Beyer [a,2], Mark Coburn [b,*,2]

[a] Institute for Neuroanatomy, Medical Faculty, RWTH Aachen, Wendlingweg 2, D-52074 Aachen, Germany
[b] Department of Anesthesiology, University Hospital Aachen, RWTH Aachen, Pauwelsstr. 30, D-52074 Aachen, Germany
[c] Institute of Immunology, University Hospital Aachen, RWTH Aachen, Pauwelsstr. 30, D-52074 Aachen, Germany
[d] Department of Neurosurgery, Klinikum rechts der Isar, TU Munich, Ismaninger Str. 22, D-81675 Munich, Germany

ARTICLE INFO

Article history:
Received 6 July 2011
Received in revised form 7 October 2011
Accepted 30 October 2011
Available online 7 November 2011

Keywords:
Noble gas
Argon
Brain
Mitogen-activated protein kinase

ABSTRACT

Recently, the noble gas argon has been identified as a potent neuroprotective agent, but little is known about its cellular effects. In this *in vitro* study, we investigated argon's influence on the extracellular signal-regulated kinase (ERK) 1/2, a ubiquitous enzyme with numerous functions in cell proliferation and survival. Primary neuronal and astroglial cell cultures and the microglial cell line BV-2 were exposed to 50 vol.% argon. Further possible effects were studied following stimulation of microglia with 50 ng/ml LPS. ERK 1/2 activation was assessed by phosphorylation state-specific western blotting, cytokine levels by real-time PCR and western blotting. Total phosphotyrosine phosphatase activity was examined with p-nitrophenylphosphate. After 30 min exposure, argon significantly activated ERK 1/2 signaling in microglia. Enhanced phosphorylation of ERK 1/2 was also found in astrocytes and neurons following argon exposure, but it lacked statistical significance. In microglia, argon did not substantially interfere with LPS-induced ERK1/2 activation and inflammatory cytokine induction. Addition of the MEK-Inhibitor U0126 abolished the induced ERK 1/2 phosphorylation. Cellular phosphatase activity and the inactivation of phosphorylated ERK 1/2 were not altered by argon. In conclusion, argon enhanced ERK 1/2 activity in microglia via the upstream kinase MEK, probably through a direct mode of activation. ERK 1/2 signaling in astrocytes and neurons *in vitro* was also influenced, although not with statistical significance. Whether ERK 1/2 activation by argon affects cellular functions like differentiation and survival in the brain *in vivo* will have to be determined in future experiments.

1. Introduction

Though chemically inert, the biological activity of noble gases has been substantially documented (Behnke and Yarbrough, 1939; Lawrence et al., 1946). Numerous studies focused on xenon due to its favorable anesthetic profile (Coburn et al., 2005; Rossaint et al., 2003). In addition, xenon has been shown to exert organ protective properties in various *in vitro* and *in vivo* models of tissue injury (Coburn et al., 2008; Ma et al., 2009; Mio et al., 2009; Weber et al., 2006a). The element argon, atomic number 18, is the most abundant noble gas in the atmosphere with a fraction of 0.93% (Riedel, 1990; Ruzicka et al., 2007). In contrast to xenon, argon does not produce anesthesia at atmospheric pressure; it anesthetizes both humans and animals only at elevated pressures (Behnke and Yarbrough, 1939; Koblin et al., 1998; Ruzicka et al., 2007). Nevertheless, comparable to xenon recent findings have also demonstrated a beneficial, protective effect of argon on neurons (Jawad et al., 2009) and neuronal tissue under ischemic conditions (Yarin et al., 2005; Loetscher et al., 2009;) *in vitro* and on hypoxic cardiac and brain tissue *in vivo* (Pagel et al., 2007; Ryang et al., 2011). The underlying molecular mechanisms for these protective properties of argon are as yet unknown.

The extracellular signal-regulated kinase (ERK) 1/2, a ubiquitous member of the mitogen-activated protein kinase (MAPK) family, is one of the key kinases in cellular signal transduction. Depending on the activating stimulus, it promotes a diversity of functions ranging from activation of gene transcription programs to cell proliferation, and cell differentiation. Meanwhile ERK 1/2 activation has been attributed a pro-survival role in most cell types, it is also involved in neuronal cell death (Subramaniam and Unsicker, 2010). In immune cells, ERK 1/2 signaling plays an important role in inflammatory

* Corresponding author at: Department of Anesthesiology, University Hospital Aachen, Pauwelsstr. 30, D-52074 Aachen, Germany. Tel.: +49 241 8035394; fax: +49 241 8082406.
E-mail address: mcoburn@ukaachen.de (M. Coburn).
[1] Present address: Department of Anesthesiology, University Hospital Aachen, RWTH Aachen, Pauwelsstr. 30, D-52074 Aachen, Germany.
[2] These two authors contributed equally to the work as senior authors.

0014-2999/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2011.10.045

activation and modulation of the lipopolysaccharide (LPS)-mediated cytokine response (Guha and Mackman, 2001; Jung et al., 2010; Rousseau et al., 2008; Scherle et al., 1998).

There is growing evidence that noble gases interfere with cellular signaling. Xenon exposure promoted the activation of p38 MAPK and ERK 1/2 pathways in different *in vitro* and *in vivo* models (Fahlenkamp et al., 2011; Weber et al., 2005, 2006a). ERK 1/2 activation was also noted after helium preconditioning in rabbit hearts (Pagel et al., 2007). Inhibition of ERK 1/2 signaling nullified the cardioprotective effects of xenon and helium (Pagel et al., 2007; Weber et al., 2006a). A stimulation of the ERK 1/2 pathway also seems to exert a superimposing effect of xenon on LPS-induced cytokine expression (De Rossi et al., 2004; Fahlenkamp et al., 2011).

The aim of the present study was to investigate the selective influence of argon on ERK 1/2 signaling in microglia using the microglial cell line BV-2. Corresponding to the effects observed with xenon (De Rossi et al., 2004; Fahlenkamp et al., 2011), the cytokine response after argon exposure was furthermore assessed during LPS-stimulation. Astroglia and neuronal cell cultures were employed to compare results from the microglial cell line experiments to further brain cell types.

2. Materials and methods

If not otherwise stated, chemicals were purchased from Roth, Karlsruhe, Germany. Bacterial lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) was obtained from Sigma-Aldrich (Steinheim, Germany). All buffers were prepared with purified water (Milli-Q UF plus system, Millipore, Bedford, MA, USA).

2.1. Cell culture and treatment

2.1.1. Animals

BALB/c mice were purchased from Charles River Laboratories GmbH (Sulzfeld, Germany). All procedures were performed in compliance with the local Institutional Ethical Review Committee and have been approved by the animal protection representative at the Institute of Animal Research at the RWTH Aachen University Hospital, according to the German animal protection law §4, Section 3.

2.1.2. Primary neuronal cultures and treatment

Primary cortical neurons were prepared from cortices of BALB/c mouse brains at gestation day 14/15 and cultured as described previously with some modifications (Singh et al., 2010). Briefly, mice embryos were taken from pregnant females after cervical dislocation. Brain cortices were isolated and the meninges removed. Subsequently, the tissue was mechanically dissociated and digested in the presence of 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) for 15 min at 37 °C. The suspension was supplemented with 20% heat-inactivated fetal bovine serum (FBS; PAA, Coelbe, Germany) and DNase I (170 U/ml; Sigma-Aldrich, Munich, Germany), minced with a Pasteur pipette, and centrifuged for 5 min at 1000 g. Cells were re-suspended in neurobasal medium (NBM, Invitrogen, Karlsruhe, Germany) containing 0.4% B27, 0.5% penicillin and streptomycin (Invitrogen, Karlsruhe, Germany), 2 mM L-glutamine (Glutamax®, Invitrogen, Karlsruhe, Germany), 10% FBS (PAA, Coelbe, Germany) and plated onto poly-L-ornithine-coated (Sigma-Aldrich, Steinheim, Germany) culture dishes at a density of $1.5–2.0\times10^5$ cells per cm^2. Cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After 24 h, the NBM was depleted of FBS. This procedure typically yields neuron-enriched cultures containing >90% neurons and <10% glial cells, mostly astrocytes.

Sub-confluent primary neuron cell cultures in fresh NBM were exposed to a humidified 50 vol.% argon gas mixture (50 vol.% argon, 24 vol.% nitrogen, 21 vol.% oxygen, 5 vol.% CO_2; Air Liquide, Krefeld, Germany) at 37 °C for 15, 30, 60 and 120 min. Cells of the same preparation maintained in a humidified atmosphere (74 vol.% nitrogen, 21 vol.% oxygen, 5 vol.% CO_2) under the same conditions served as controls.

2.1.3. Primary astrocytes cultures and treatment

Astroglial cultures were prepared from postnatal day 1 BALB/c mice as previously described (Misiak et al., 2010). Briefly, brains from decapitated mice were removed and transferred into preparation buffer consisting of 10 mM HEPES, 154 mM NaCl, 10 mM glucose, 2 mM KCl, and 15 μM bovine serum albumin. The cerebral cortex was dissected and after removal of the meninges incubated in PBS containing 0.1% trypsin and 0.02% EDTA for 15 min. Subsequently, the tissue was minced with a Pasteur pipette and filtered through a 50 μm nylon mesh. The cells were centrifuged at 300 *g* for 5 min, suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 16% FBS (PAA, Coelbe, Germany), 0.5% penicillin and streptomycin (Invitrogen, Karlsruhe, Germany), 0.25% Fungizone (Invitrogen, Karlsruhe, Germany), 2 mM L-glutamine (Glutamax®, Invitrogen, Karlsruhe, Germany) and plated onto poly-L-ornithine-coated (Sigma-Aldrich, Munich, Germany) culture dishes at a density of $1–2\times10^6$ cells per cm^2. Astrocyte cultures were grown at 37 °C in a humidified atmosphere of 95 vol.% air and 5 vol.% CO_2 and once trypsinized and plated at lower density. Sub-confluent (80%) astrocytes were incubated in NBM supplemented with 0.2% B27, 0.5% penicillin and streptomycin, 0.25% Fungizone and 2 mM L-glutamine for 48 h and subsequently used for experiments.

As described for neurons, primary astrocyte cell cultures were exposed to a humidified 50 vol.% argon gas mixture (50 vol.% argon, 24 vol.% nitrogen, 21 vol.% oxygen, 5 vol.% CO_2; Air Liquide, Krefeld, Germany) at 37 °C for 15, 30, 60 and 120 min. Cells of the same preparation, maintained in a humidified atmosphere (74 vol.% nitrogen, 21 vol.% oxygen, 5 vol.% CO_2) under the same conditions, served as controls.

2.1.4. Microglia culture and treatment

The BV-2 cell line, an immortalized murine microglia cell line (Bocchini et al., 1992), was cultivated as described before (Fahlenkamp et al., 2011). Briefly, cells were maintained in a humidified atmosphere containing 5 vol.% CO_2 at 37 °C in DMEM (Invitrogen, Karlsruhe, Germany) with 10 vol.% FBS (PAA, Coelbe, Germany), 0.5% penicillin and streptomycin (Invitrogen, Karlsruhe, Germany).

Experiments were carried out in fresh experimental medium after 24 h incubation in serum-depleted experimental medium (DMEM lacking phenol red, 0.5% FBS, 0.5% penicillin and streptomycin). Exposure to gases was performed in a small gas chamber (volume 0.925 l) at 37 °C as described before (Coburn et al., 2008; Loetscher et al., 2009). Briefly, the chamber was flushed with a normobaric humidified gas mixture at 37 °C for 2 min at 2.5 l/min to ensure gas replacement and then was sealed. At the end of exposure time, cells were removed from the chamber and further processed.

BV-2 cells were exposed to either 50 ng/ml LPS (Fahlenkamp et al., 2011) in fresh experimental medium and humidified atmosphere (74 vol.% nitrogen, 21 vol.% oxygen, 5 vol.% CO_2; Air Liquide, Germany) or a X vol.% argon gas mixture (X vol.% argon, 74–X vol.% nitrogen, 21 vol.% oxygen, 5 vol.% CO_2; Air Liquide, Krefeld, Germany), or a combination of 50 ng/ml LPS and argon gas mixture. Cells without exposure to argon or LPS served as controls. Argon concentration and exposure time were established in a set of dose–response experiments at the beginning of the study. Fig. 1A depicts the time schedule of the experimental course.

2.2. Cell viability

Microglial vitality was assessed after 24 h of treatment (BV-2; 1.5×10^4 cells in 100 μl medium in 96-well culture plates; Falcon, USA) with the CytoTox 96® non-radioactive cytotoxicity assay (Promega, Mannheim, Germany) and the Cell Titer-Blue® cell viability assay