Neuroscience 165 (2010) 1138–1146

ACTIVATED PROTEIN C PREVENTS GLUTAMATE- AND THROMBIN-INDUCED ACTIVATION OF NUCLEAR FACTOR-κB IN CULTURED HIPPOCAMPAL NEURONS

L. GORBACHEVA,[a,b] V. PINELIS,[c] S. ISHIWATA,[d] S. STRUKOVA[a] AND G. REISER[b]*

[a]*Lomonosov Moscow State University, Department of Human and Animal Physiology, 119991, Moscow, Russia*

[b]*Institute for Neurobiochemistry, Otto-von-Guericke University, Medical Faculty, Magdeburg, Germany*

[c]*Centre for Children's Health, Russian Academy of Medical Sciences, Moscow, Russia*

[d]*Department of Physics, Faculty of Science and Engineering, Waseda University, Tokyo, Japan*

Abstract—Brain injury is associated with neuroinflammation, neurodegeneration, and also blood coagulation with thrombin formation and generation of activated protein C (APC). We have previously shown that APC, a serine protease of hemostasis, at very low concentrations has protective effects in rat hippocampal and cortical neurons at glutamate-induced excitotoxicity through protease-activated receptor-1 (PAR-1) or endothelial receptor of protein C (EPCR)/PAR-1. The transcription factor nuclear factor κB (NF-κB) takes part in regulating neuronal survival in several pathological conditions. To elucidate the impact of NF-κB in APC-mediated cell survival, we investigated nuclear translocation of NF-κB p65 at glutamate- or thrombin-induced toxicity in hippocampal neurons. We used immunoassay and immunostaining with confocal microscopy with anti-NF-κBp65 antibody. We show that APC at concentrations as low as 1–2 nM inhibits translocation of NF-κB p65 into the nucleus of cultured rat hippocampal neurons, induced by 100 μM glutamate or 50 nM thrombin (but not 10 nM). The blocking effect of APC on NF-κB p65 translocation was observed at 1 and 4 h after treatment of neurons with glutamate, when the NF-κBp 65 level in the nucleus was significantly above the basal level. Then we investigated whether the binding of APC to EPCR/PAR-1 is required to control NF-κB activation. Antibodies blocking PAR-1 (ATAP2) or EPCR (P-20) abolished the APC-induced decrease of nuclear level of NF-κB p65 at glutamate-induced toxicity, whereas control antibodies to PAR-1 (S-19) and EPCR (IgG) exerted no effect. Thus, we suggest that the activation of NF-κB in rat hippocampal neurons mediates the glutamate- and thrombin-activated cell death program, which is reduced by exposure of cells to APC. APC induces the reduction of the nuclear level of NF-κB p65 in hippocampal neurons at glutamate-induced excitotoxicity via binding to EPCR and subsequent PAR-1 activation and signaling.

*Corresponding author. Tel: +49-0-391-67-13088; fax: +49-0-391-67-13097.
E-mail address: georg.reiser@med.ovgu.de (G. Reiser).
Abbreviations: APC, activated protein C; EPCR, endothelial protein C receptor; Glu, glutamate; HBS, HEPES-buffered saline; LDH, lactate dehydrogenase; NF-κB, nuclear factor κB; NMDA, N-methyl-D-aspartate; PAR, protease-activated receptor; PC, protein C.

Key words: excitotoxicity, neurons, activated protein C, thrombin, nuclear factor κB, protease-activated receptor.

Activated protein C (APC) is a serine protease derived from its precursor, protein C (PC). APC is generated on the cell surface by cleavage of an activation peptide by thrombin bound to thrombomodulin (Esmon, 2006). PC was thought to be synthesized only in the liver, but recently PC expression was shown in vascular endothelial cells and skin keratinocytes of epidermis (Xue et al., 2007a; Jackson and Xue, 2008). The conversion of PC to APC is augmented by endothelial PC receptor (EPCR), which is present on endothelial cells, hematopoietic stem cells, various leukocytes, human vascular smooth muscle cells and skin keratinocytes (Xue et al., 2005; Balazs et al., 2006; Bretschneider et al., 2007; Xue et al., 2007b). Blood vessels and the epidermis possess their own individual PC systems, which can synthesize PC, activate PC to APC to trigger the receptor-mediated functions of APC.

APC has a well characterized anticoagulant activity, directed to the down-regulation of thrombin formation in a negative feedback loop via inhibition of activated Factors Va and VIIIa (Esmon, 2006). More recently, APC was shown to have anti-inflammatory and cytoprotective properties, which are independent of its anticoagulant activity, and an engineered APC mutant was made with markedly reduced anticoagulant activity, but unaltered cytoprotective activities (Mosnier and Griffin, 2006; Mosnier et al., 2007a, 2009).

APC exerts a direct protective effect via either EPCR-dependent activation of protease-activated receptor-1 (PAR-1) on endothelial cells (Cheng et al., 2003; Finigan et al., 2005; Riewald and Ruf, 2005) or via PAR-1 and PAR-3 on mouse cortical neurons treated by two inducers of apoptosis, N-methyl-D-aspartate (NMDA) and staurosporine (Guo et al., 2004). Moreover, neuroprotective effects of APC can be found *in vivo* (Guo et al., 2004; Mosnier et al., 2007b). A neuroprotective effect of APC on motor neurons was demonstrated after transient ischemia in spinal cord of rabbits (Yamauchi et al., 2006). APC can block tissue plasminogen activator-mediated brain hemorrhage after transient brain ischemia and embolic stroke in rodents (Cheng et al., 2006). The mechanism underlying the pro-survival effects of APC at brain ischemia and neurotoxicity is currently unknown, but is presumed to be related to the ability of APC to prevent inflammation and to inhibit apoptosis of brain endothelial cells through transcriptional in-

0306-4522/10 $ - see front matter © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2009.11.027

hibition of the tumor suppressor protein p53 (Cheng et al., 2003).

In our previous study, we have demonstrated that the serine proteases of hemostasis—thrombin, Factor Xa and APC, at very low concentrations, exert protective effects on primary cultures of hippocampal and cortical neurons at glutamate (Glu)-induced excitotoxicity (Kiseleva et al., 2004; Gorbacheva et al., 2005, 2008; Strukova et al., 2005, 2006). Overstimulation of Glu receptors, described as the excitotoxicity phenomenon, is a most important mechanism of neurodegeneration in brain stroke, brain trauma, in Alzheimer's and Parkinson's and other neurodegenerative diseases (Hossain, 2005). The involvement of PAR-1 and PAR-4 and their activating protease thrombin in neurodegenerative diseases has been reviewed recently (Sokolova and Reiser, 2008).

There is a growing body of evidence demonstrating that the transcription factor nuclear factor-κB (NF-κB) signaling contributes to ischemic brain damage (Schwaninger et al., 2006; Ridder and Schwaninger, 2009). Moreover, there are data that glutamate excitotoxicity is associated with an increase in NF-κB DNA-binding (Schölzke et al., 2003; Ishige et al., 2005; Cherng et al., 2006).

The NF-κB family of transcription factors consists of five members, p50, p52, p65 (RelA), c-Rel, and RelB, which have an N-terminal Rel homology domain responsible for DNA binding and homo- and heterodimerization. The transcription activation domain necessary for the positive regulation of gene expression is present only in p65, c-Rel, and RelB. In its inactive state, NF-κB dimers are associated with one of three typical IκB (inhibitor of NF-κB) proteins, IκBα, IκBβ, or IκBε, or the precursor proteins of p52 (p100) or of p50 (p105) (Hayden and Ghosh, 2008). IκB proteins bind NF-κB p50/p65 complexes in the cytosol and block their activation. Phosphorylation of IκB activates NF-κB that causes dissociation of IκB from the p50/p65 complex. Subsequently, the activated p50/p65 complex enters the nucleus and functions as a transcription factor. NF-κB regulates the expression of genes involved in a variety of cellular processes, including cell survival and inflammation (Mattson et al., 2000; Gutierrez et al., 2005).

NF-κB was shown on endothelial cells and monocytes to be required for APC-mediated anti-apoptotic and anti-inflammatory signalling. The action of APC on endothelial cells is known to be exerted via the blockade of NF-κB translocation to the nucleus and inhibition of the expression of the pro-inflammatory agents (Riewald and Ruf, 2005; Mosnier et al., 2007b). In a recent study it was shown that in endothelial cells under the conditions, when EPCR is occupied by PC, low concentrations of thrombin can inhibit the NF-κB pathway in response to tumor necrosis factor-α (Bae and Rezaie, 2009). APC inhibits NF-κB signaling in monocytes (Joyce et al., 2001; Yuksel et al., 2002).

In the nervous system, NF-κB is widely expressed (Bhakar et al., 2002; Yalcin et al., 2003; Kaltschmidt et al., 2005) and is activated by a variety of neurotrophic factors, cytokines and neurotransmitters (Mattson et al., 2000; Yalcin et al., 2003; Pizzi and Spano, 2006). The impact of APC on the regulation of NF-κB signaling in neurons at Glu-induced toxicity is currently unknown.

We here show that APC via PAR-1 or the cooperation of PAR-1 with EPCR modulates rat hippocampal neuron survival at Glu-induced excitotoxicity through regulation of NF-κB activation. We suggest that APC via PAR-1 or PAR-1/EPCR can prevent the NF-κB activation at excitotoxicity, thus mediating cell survival due to inhibition of p65 translocation into the nucleus. We expect that APC plays the critical role of the endogenous regulator of signaling pathways that control nerve cells survival at excitotoxicity.

EXPERIMENTAL PROCEDURES

Hippocampal neuronal culture

Primary cultures of neurons from hippocampus were obtained from brain of 1–3 day-old Wistar rats. The cell suspension was obtained according to our previously described technique (Gorbacheva et al., 2006) and plated on dishes coated with ethyleneimine polymer solution (1 mg/ml). The cells were allowed to sediment for 1 h at 37 °C and 5% CO_2, then non-attached cells were removed and 1.5 ml of culture medium (neurobasal medium A containing 2% Supplement B-27 and 0.5 mM L-glutamine) was added. At day 3, cytosine arabinoside (AraC, 10^{-5} M) was added to suppress the growth of glial cells. For experiments, cells were used at days 9–10 in culture. All experimental procedures conformed to the "Guidelines for Proper Control of Animal Experiments" approved by the local ethics regulations.

Cell exposure to glutamate and thrombin

Cells were incubated for 15 min with or without 1–2 nM APC from human plasma (Sigma, Taufkirchen, Germany) in HEPES-saline buffer (in mM: NaCl, 145; KCl, 5; $CaCl_2$, 1.8; glycine, 0.01; glucose, 5; HEPES, 20; pH 7.4) and then exposed for 30 min to 100 μM glutamate or for 45 min to thrombin (bovine; Sigma) at various concentrations in the same buffer. To analyze the role of PAR-1 and EPCR, cells were pretreated for 30 min with blocking or control antibodies to these receptors, before we added APC and glutamate. The following antibodies purchased from Santa Cruz Biotechnology (Heidelberg, Germany) were used: a cleavage-site blocking anti-PAR-1 antibody (ATAP2, 20 μg/ml), a binding-site blocking anti-EPCR antibody (P-20, 10 μg/ml). Control antibodies c-PAR-1 (S-19) and c-EPCR (IgG) were used at concentrations of 20 μg/ml and 10 μg/ml, respectively. After the exposure, cultures were supplemented with culture medium.

LDH assay

Neuronal cell death was estimated by lactate dehydrogenase (LDH) release after 24 h. LDH release was detected using a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany). The percentage of neuronal cell death was calculated as (LDH activity in cell medium/total LDH content)×100, where total LDH content was determined after cell lysis (0.2% Triton X-100 for 10 min at 37 °C).

Immunocytochemistry

Neurons were fixed with 4% paraformaldehyde (in phosphate buffer, containing 4% sucrose) for 25 min at room temperature and permeabilized with 0.3% Triton X-100 in blocking buffer (16.7% fetal calf serum, 450 mM NaCl and 20 mM phosphate buffer). Then cells were incubated overnight at 4 °C with primary antibodies against NF-κB p65 (C22B4; 1:100). After that, cells were washed three times with high salt buffer (phosphate buffer,