FEBS Letters 543 (2003) 170–173 FEBS 27261

Down-regulation of ARC contributes to vulnerability of hippocampal neurons to ischemia/hypoxia

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Received 8 April 2003; accepted 10 April 2003

First published online 1 May 2003

Edited by Jesus Avila

Abstract ARC is a caspase recruitment domain-containing molecule that plays an important role in the regulation of apoptosis. We examined ARC expression during neuronal cell death following ischemic injury in vivo and in vitro. After exposure to transient global ischemic conditions, the expression of ARC was substantially reduced in the CA1 region of hippocampus in a time-dependent manner with concomitant increase of TUNEL-positive cells. Quantitative analysis using Western blotting exhibited that most of ARC protein disappeared in the cultured hippocampal neurons exposed to hypoxia for 12 h and showing 60% cell viability. Forced expression of ARC in the primary cultures of hippocampal neurons or B103 neuronal cells significantly reduced hypoxia-induced cell death. Further, the C-terminal P/E rich region of ARC was effective to attenuate hypoxic insults. These results suggest that down-regulation of ARC expression in hippocampal neurons may contribute to neuronal death induced by ischemia/hypoxia.

Key words: Apoptosis repressor with caspase recruitment domain; Ischemia; Hypoxia; Hippocampal neuron; Rat brain

1. Introduction

Degeneration of neurons is the fundamental process responsible for the clinical manifestations of many different neuropathological disorders, including Alzheimer's disease, Parkinson's disease, and epilepsy [1,2]. The neurodegeneration mechanism in such disorders is not well known. However, one of the principal pathways controlling cell death is the regulation of protein–protein interaction, such as the death domain, the death effector domain, and the caspase recruitment domain (CARD) [3–5].

ARC (apoptosis repressor with CARD) is an apoptotic regulatory protein and expressed in a majority of myocytes in the heart and skeletal muscle. It contains two domains: C-terminal P/E rich domain and N-terminal CARD that is linked to caspase inhibition, such as caspase-2 and -8 [6]. Recent studies reported that overexpression of ARC inhibited hypoxia- and oxidant stress-induced cell death in the cardiac myocytes by preserving mitochondria function [6,7], while a proapoptotic role of ARC was also reported in serum withdrawal-induced death of PC12 cells [8]. Alternative splicing variants of ARC and cell death-induced subcellular translocalization of ARC to intracellular membrane or nuclei suggest their diverse roles in the death signaling events [9,10]. However, the mechanism by which ARC interferes with the cell death is not clearly defined yet.

In the present study, we have examined the regulation of ARC expression after transient global cerebral ischemia in rat brain and found the protective role of ARC expression in the cultured hippocampal neurons during hypoxia.

2. Materials and methods

2.1. Transient global cerebral ischemia in rats and immunostaining

All animal experiments were performed in accordance with the Guide of Ulsan University College of Medicine for Care and Use of Laboratory Animals. Under halothane anesthesia, common carotid arteries of male Sprague–Dawley rats weighing 300–350 g were bilaterally occluded with vascular clamps and mean arterial blood pressure was lowered to 50 ± 5 mm Hg by withdrawing blood from the femoral artery. After 12 min, the blood perfusion was recovered by removing clips and re-infusing the blood. At 24 and 72 h after ischemia, brains were removed and quickly frozen in liquid nitrogen. Coronal brain sections containing hippocampi were prepared with cryostat (thickness, 10 μm) and put onto the slide glass pre-coated with poly-L-lysine (Sigma). After incubation with anti-ARC antibody, sections were incubated with avidin–biotin complex (ABC kit, Santa Cruz Biotech.). To identify neuronal death, the brain sections were stained with TUNEL assay (Oncor).

2.2. Cell culture

Primary hippocampal cell cultures were dissected from gestation stage E17 of rat. Hippocampi were incubated at 37°C for 15 min in trypsin/EDTA (Gibco BRL) and triturated with a glass pipette to dissociate cells. After centrifugation at 2000 rpm for 2 min, the pellet was resuspended and plated at the density of 3×10^5 cells/ml in four-well plates previously coated with poly-L-lysine in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) and 5% horse serum (Equitech-bio, Inc.). Cells were allowed to attach for 45 min at 37°C with 5% CO_2 and cultured with serum-free neurobasal medium with B27 supplement, 0.5 mM L-glutamine, and penicillin/streptomycin for 5 days (all from Gibco BRL). B103 cells were cultured in DMEM supplemented with 10% FBS.

2.3. Hypoxia induction and cell viability assay

Hypoxia was achieved by culturing the cells in serum- and glucose-deficient salt solution (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1 mM $NaH_2PO_4 \cdot H_2O$, 1.8 mM $CaCl_2$, 26 mM $NaHCO_3$) in an

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Abbreviations: CARD, caspase recruitment domain; ARC, apoptosis repressor with CARD; GFP, green fluorescence protein

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00444-7

air-tight plexiglas chamber (BBL™, Becton Dickinson) for 0–24 h at 37°C as previously described [11]. Cells were counted before and after exposure to hypoxia and stained with trypan blue to evaluate cell viability. The mortality of the neurons transfected with green fluorescence protein (GFP)-fusion construct was assessed as a percentage of trypan blue-positive per fluorescence-positive staining.

2.4. DNA construction

*Eco*RI and *Bam*HI fragments encoding full-length (amino acids 1–208), N-terminal domain of ARC (amino acids 1–98), or C-terminal domain (amino acids 99–208) were amplified by polymerase chain reaction and subcloned into pEGFP-N1.

2.5. DNA transfection and stable cell line generation

After culturing for 5 days, hippocampal neurons were transfected with 1 μg of control vector (pEGFP) or ARC expression plasmids using LipofectAMINE PLUS™ Reagent according to the protocol provided by Gibco BRL. After 5 h, the transfected neurons were replaced in neurobasal medium supplemented with B27 and glutamine (all from Gibco BRL). B103 cells were transfected with pEGFP or pEGFP-ARC using LipofectAMINE PLUS™ and replaced in DMEM/10% FBS with G418 sulfate (800 μg/ml, Gibco BRL) for 3 weeks. Colonies derived from a single cell were picked and expanded as stable cell lines.

2.6. Western blotting

Cell extracts (20 μg) prepared from primary cultures or cell lines exposed to hypoxia were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Proteins were detected using primary antibodies, horseradish peroxidase-linked secondary antibody, and enhanced chemiluminescence system (ECL™) (Amersham-Pharmacia Biotech.).

3. Results

3.1. Reduced expression of ARC in the rat brain after transient global ischemia

To examine whether ARC was implicated in ischemic insult of rat brain, the expression of ARC was investigated in the hippocampus of normal and ischemic brain (Fig. 1). A coronal section of hippocampus was stained with anti-ARC antibody (Fig. 1a–c) or TUNEL assay (Fig. 1d–f, small box). An intensive and strong cellular expression pattern of ARC was observed in hippocampus of sham-operated rats (Fig. 1a).

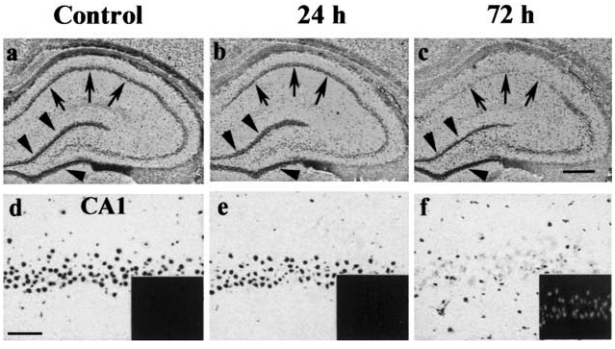

Fig. 1. Correlation between ARC expression and neuronal cell death in the hippocampus after transient global ischemia. Upper panel, low-magnification photographs of anti-ARC antibody-stained hippocampi of sham-operated rat (a) and rat at 24 h (b) and 72 h (c) after reperfusion. Arrowheads indicate dentate gyrus. Scale bar, 200 μm. Lower panel, high-power photographs of CA1 pyramidal neurons (arrows, upper panel) stained with anti-ARC antibody and showing reduction of ARC immunoreactivity. Typical pycnotic bodies were observed in most of the neurons at 72 h (f) compared to sham control (d) or 24 h after ischemic damage (e) (small boxes, TUNEL staining). Scale bar, 100 μm.

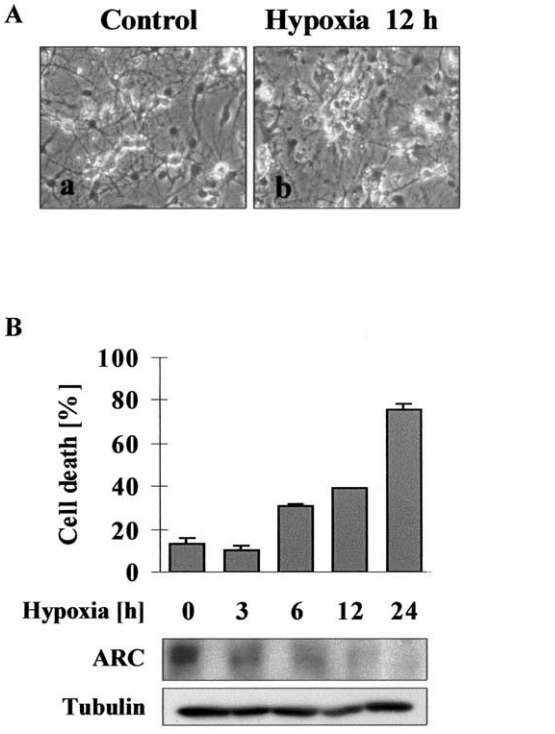

Fig. 2. Quantitative analysis of ARC expression in the cultured hippocampal neurons under hypoxic insults. Cultured hippocampal neurons were left untreated (control) or exposed to hypoxic insult for the indicated times. Cell viability was determined by trypan blue exclusion assay. The morphology of neurons (A), neuronal cell death (B, upper panel), and Western blotting (B, lower panel) were assessed.

After transient global ischemia, the ARC protein level clearly decreased at 24 h and almost disrupted at 72 h in the CA1 and CA2 regions of the hippocampus (Fig. 1b,c, arrows), but not in dentate gyrus (Fig. 1b,c, arrowheads). The numbers of ARC-positive pyramidal neurons in the CA1 of hippocampus were less in response to ischemia than control (Fig. 1d–f). In contrast, TUNEL assay showed that apoptosis-positive neurons were observed at 72 h after induction of ischemia (Fig. 1d–f, small boxes), indicating that down-regulation of ARC in the hippocampal neurons damaged by ischemic insult inversely correlates with the appearance of dying cells.

3.2. Reduced expression of ARC in cultured hippocampal neurons after hypoxia

ARC expression was further assessed in the cultured hippocampal neurons undergoing hypoxia-induced neuronal death. Primary hippocampal neurons were prepared from gestation stage E17 of rat and exposed to hypoxia for the indicated times. Hypoxic neurons were evident at 12 h with the morphological characteristics by the appearance of a rough, darkened soma and loss of phase brightness (Fig. 2A,b), while viable neurons were round to oval and showed smooth soma and intact neurites (Fig. 2A,a). Approximately 50% of neurons were stained by trypan blue at 12 h after induction of hypoxia and 80% of hypoxic neurons were evident at 24 h compared to control neurons (Fig. 2B). Western blot analysis showed that the expression level of ARC decreased in a time-dependent manner during hypoxia. Approximately 50% of ARC protein level was apparently reduced in the neurons

exposed to hypoxia for 6 h and most ARC protein disappeared at 24 h compared to control cultures, indicating that ARC protein disappears in dying hippocampal neurons.

3.3. Hippocampal neurons expressing ARC are resistant to hypoxia-induced cell death

To examine whether the reduction of ARC protein is functionally associated with ischemia-induced death of hippocampal neurons, the protective function of ARC expression was addressed by gene-transfer analysis. The cultured hippocampal neurons were transiently transfected with expression plasmid encoding full-length ARC fused to GFP or control DNA. Two days after DNA transfection, the vulnerability to neuronal death triggered by hypoxia was analyzed based on the morphology and trypan blue staining of the GFP-positive neurons. Under normoxia, the viabilities of hippocampal neurons expressing ARC-GFP were similar with those of the control neurons (Fig. 3A). On the contrary, the hippocampal neurons transfected with full-length ARC exhibited significantly increased viability in response to hypoxia, compared to control neurons. When the neurons were exposed to hypoxia for 10 h, the mortality of neurons expressing ARC was higher than that of the control neurons; the viability was increased from 53% to 80%. These results indicate that ARC suppresses hypoxia-induced neuronal death.

Deletion mutants containing either only the N-terminal CARD or the C-terminal P/E rich domain of ARC were generated to define a domain responsible for the suppression of hypoxia-induced cell death. Hippocampal neurons transiently expressing the C-terminal P/E rich domain of ARC became morphologically shrunk at 5 h after hypoxia but were desensitized to hypoxia as examined with trypan blue staining (Fig. 3B). In contrast, the N-terminal CARD of ARC showed a different cellular distribution pattern from the C-terminal P/E domain, localized mainly in the cell body but not in the neurites of the transfected neurons and upon hypoxic condition, did not show such inhibitory effects as full-length ARC or the C-terminal P/E domain. These results suggest that the inhibitory activities of ARC during ischemia/hypoxia reside in the C-terminal P/E rich domain of ARC.

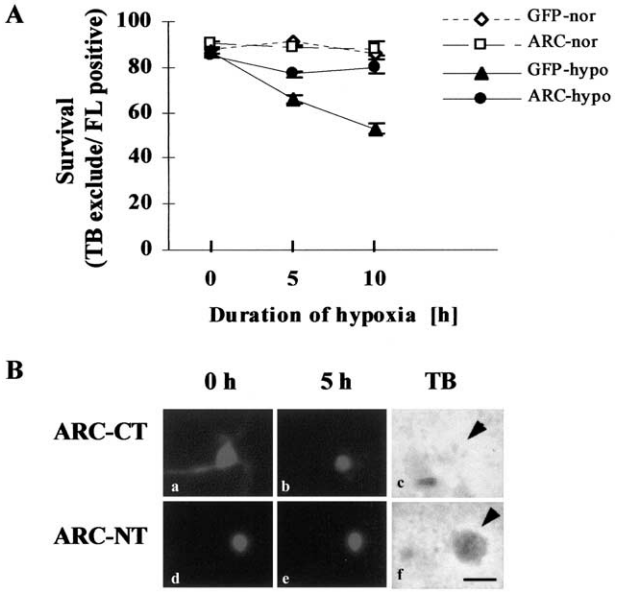

Fig. 3. Transient expression of the C-terminal P/E rich domain of ARC protects hippocampal neurons from hypoxia-induced cell death. A: Hippocampal neurons were transiently transfected with pEGFP or pEGFP-ARC, and then exposed to hypoxia for the indicated times. The mortality of the GFP-positive neurons was scored using trypan blue staining. Bars depict means ± S.E.M. from three independent experiments (total number of the transfected neurons, 20–30 cells/transfection). B: The hippocampal neurons expressing either the C-terminus (ARC-CT) or the N-terminus of ARC (ARC-NT) were exposed to hypoxia for 5 h and stained with trypan blue (TB, arrowheads). The cells were visualized under a fluorescence microscope. Scale bar, 20 μm.

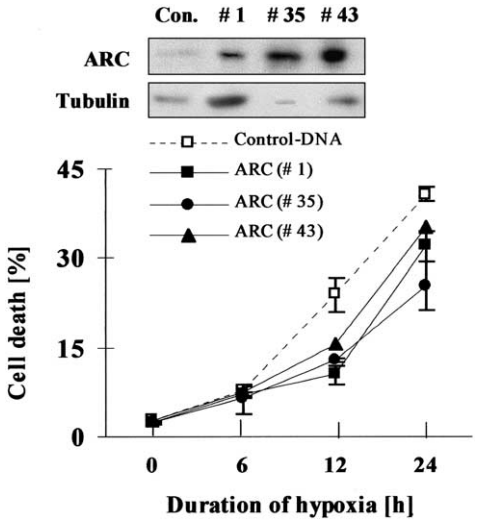

Fig. 4. Stable expression of ARC inhibits hypoxia-induced death of B103 neuronal cells. B103 cells were permanently transfected with pcDNA (control) or pcDNA-ARC, and examined for the expression level of ARC with Western blot analysis (upper panel). Three different stable clones (#1, #35, and #43) were exposed to hypoxia for the indicated times and cell viability was determined (lower panel). Values represent the means ± S.E.M. of three separate experiments.

3.4. B103 neuronal cells overexpressing ARC reduce hypoxia-induced cell death

To further examine the inhibitory role of ARC in hypoxia-induced neuronal cell death, we generated B103 neuronal cell lines overexpressing full-length ARC or control DNA. We selected for study three clones of B103 cells stably overexpressing different levels of ARC and then analyzed the vulnerability of those cells to hypoxia (Fig. 4). Forced expression of ARC in B103 cells significantly decreased hypoxia-induced cell death in comparison with the vector-transfected control cells, while ARC itself did not affect the basal level of cell viability (Fig. 4), confirming that ARC expression reduces death of neuronal cells exposed to hypoxia.

4. Discussion

Transient global ischemic insults in the brain can result in the selective death of certain neurons, for example, the pyramidal neurons in the CA1 region of the hippocampus. In the present study, we demonstrate for the first time that ARC protein is also detected in the hippocampal neurons and its expression level is reduced in hippocampal neurons following ischemic insults. In addition, the protective effects of ARC overexpression on hypoxic insults enabled us to propose that ARC protein was reduced in the affected neurons as a necessary step leading to neuronal death during ischemia/hyp-

oxia. These results are reinforced by the previous observation that in cardiac cell lines, ARC exerted protective properties in hypoxia-induced apoptosis [7].

Under ischemic damage, plummeting of cellular ATP level impairs membrane ion-motive ATPases to remove Na^+ and Ca^{2+} from the cell, allowing membrane depolarization and promoting activation of synaptic glutamate receptors for massive calcium influx and the downstream activation of deleterious events including the activation of catabolic enzymes and over-production of free radicals [12–14]. The mechanism by which ARC inhibits neuronal cell death remains unclear. However, several mechanisms can be proposed to explain how ARC protects neuronal cells from ischemic insults. ARC may affect the activity of the molecule involved in the apoptosis cascade, such as caspases [15,16] or members of the Bcl-2 family [17] that contributed to ischemic brain damage as evidenced by pharmacological or genetic manipulations. ARC as an interacting molecule with caspase-8 may inhibit ischemia/hypoxia-mediated activation of caspase as initially proposed by Koseki and coworkers in skeletal muscle [6]. Also, ARC may block caspase-independent events associated with apoptosis, such as hydrogen peroxide-induced cytochrome *c* release [18].

Alternatively, the notion that the C-terminal P/E rich domain of ARC inhibited ischemia/hypoxia-induced neuronal death led us to propose that the P/E rich domain, but not CARD region, functions to interfere cell death. We found that the C-terminal P/E domain was able to bind to calcium and affect intracellular calcium signaling (D. Jo and Y. Jung, manuscript in preparation). Given the importance of cytosolic free calcium during ischemia/hypoxia, the P/E domain of ARC may sequester cytosolic free calcium and protect neuronal cells from ischemic damage. Thus, the ARC level is important for the regulation of ischemia/hypoxia-mediated damage and the reduction of ARC expression in the ischemic brain in vivo may increase neuronal cell death. Further understanding of the inhibitory mechanism controlling neuronal death during ischemia needs to be established.

Acknowledgements: Y.-M.H. was partially supported by Brain Korea 21. This work was supported by a grant from the National Research Laboratory program (to Y.-K.J.) and Life Phenomena and Function Research Group Program (2000) of the Ministry of Science and Technology.

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