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RESEARCH ARTICLE

Overexpression of kynurenic acid in stroke: An endogenous neuroprotector?

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ABSTRACT

It is known that kynurenic acid (KYNA) exerts a neuroprotective effect against the neuronal loss induced by ischemia; acting as a scavenger, and exerting antioxidant action. In order to study the distribution of KYNA, a highly specific monoclonal antibody directed against KYNA was developed. This distribution was studied in control rats and in animals in which a middle cerebral artery occlusion (stroke model) was induced. By double immunohistochemistry, astrocytes containing KYNA and GFAP were exclusively found in the ipsilateral cerebral cortex and/or striatum, at 2, 5 and 21 days after the induction of stroke. In control animals and in the contralateral side of the stroke animals, no immunoreactivity for KYNA was found. Under pathological conditions, the presence of KYNA is reported for the first time in the mammalian brain from early phases of stroke. The distribution of KYNA matches perfectly with the infarcted regions suggesting that, in stroke, this overexpressed molecule could be involved in neuroprotective/scavenger/antioxidant mechanisms.

1. Introduction

Stroke is a major health problem. The Framingham Study reported that 1 of 5 women and 1 of 6 men, aged from 55 to 75 years, will experience stroke at some time during their lives. In stroke, several phases have been described (Tatro, 2006). The first is called excitotoxicity, in which a high release of glutamate occurs as well as the generation of reactive oxygen species (ROS), whereas the second phase corresponds to inflammatory activation and apoptosis. The latter phase is characterized by the activation of cytokines, caspases and both indoleamine 2,3-dioxygenase (IDO) and nitric oxide (NO) pathways. In the IDO pathway, tryptophan is catabolised, leading to the generation of nicotinamide adenine dinucleotide (NAD) and several intermediate metabolites (e.g., kynurenic acid (KYNA)) are also generated. Thus, KYNA is an intermediate molecule of the catabolism of tryptophan and it is mainly synthesized by astrocytes (Guillemin et al., 2001; López et al., 2016).

It is known that KYNA exerts a neuroprotective effect against the neuronal loss induced by ischemia (Gellért et al., 2011); that helps the scavenger activity of astrocytes, and that exerts an antioxidant activity (Lugo-Huitrón et al., 2011). Due to the beneficial mentioned roles, it is important to study the distribution and/or function of KYNA in several pathologies (e.g., stroke). In order to carry out this aim, a highly specific monoclonal antibody directed against KYNA was firstly developed. Later, and in order to study the neuroanatomical expression of KYNA in the rat central nervous system, an immunohistochemical study was carried out in normal and operated animals. In this study, we sought to investigate the alterations in KYNA expression in a rat model of stroke.

2. Material and methods

2.1. Single transient middle cerebral artery occlusion (tMCAO)

The experimental procedure of this work was performed under the guidelines of the ethics and legal recommendations of Spanish, French and European laws. This study was approved by the research commission of the University of Salamanca (Spain). Eighteen adult male Wistar rats (control: 6 animals; tMCAO procedure: 12 animals) weighing 350 gr were used. Animals were deeply anesthetized (with isoflurane (4% induction; 2.5% maintenance)) by

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means of a facial mask. As previously described (Longa et al., 1989; Qiao et al., 2009; Uluç et al., 2011), tMCAO surgical procedures were carried out. Thus, a middle cerebral artery occlusion (50–55 min) was conducted by using a rounded tip monofilament (Doccol, USA). As previously described (Uluç et al., 2011) and before the recovery from anesthesia, the analgesic buprenorphine (0.05 mg/kg) was subcutaneously administered to animals.

Once the tMCAO experimental model was performed, animals were divided into three groups: 2D, 5D and 21D. Animals belonging to the 2D group were perfused two days after the middle cerebral artery occlusion; those belonging to the 5D group, five days after the occlusion, and those of the third group (21D), twenty-one days after the surgical procedure. In each experimental group, 6 animals were used: 2 control rats and 4 tMCAO animals.

2.2. Antibody development

As previously described (Mangas et al., 2012), a primary antiserum was developed in BALB/c mice after immunization with KYNA-bovine serum albumin (BSA) immunogen linked via ethylchloroformiate (ECF). Briefly, 10 mg of KYNA were dissolved in methanol; later, 20 mg of bovine serum albumin (BSA) were dissolved in water, and 40 μl of triethylamine were added to both solutions. Then, the activation of the KYNA solution was carried out by adding an extemporaneous solution containing 375 μl of dimethylformamide mixed with 25 μl of ECF. After 10 min of activation, the solution containing KYNA was added drop by drop into a tube containing the BSA solution. Using dialysis membranes with cut-off limits between 12 and 16 KDa, the obtained conjugate (KYNA-BSA) was purified by dialysis. The purification was performed in one litre bucket at 4 °C during 36 h, changing the bath every 2–3 h (Mangas et al., 2012).

After the synthesis of KYNA-BSA, mice were immediately immunized by one injection every 2–3 weeks with the immunogen (containing KYNA-BSA). Each immunization was carried out by administering 50 μl of an immunogenic NaCl solution and 50 μl of complete (only used in the first immunization) or incomplete Freund adjuvant. After the second immunization, serum samples were collected and the antisera were pre-purified by immunoabsorption and tested by ELISA as previously described (Mangas et al., 2007, 2008, 2012). Once a highly specific polyclonal antibody was obtained, the fusion of SP2/O/Ag myeloma cells and mice splenocytes was carried out. Then, the screening and the selection of specific clones were performed. Once the highly specific monoclonal antibody against KYNA was obtained, cells were expanded in plastic flasks. Supernatant was collected every week, centrifuged and pre-purified with a saturated $(NH_4)_2SO_4$ solution, dialyzed in PBS and finally purified in an HiTrap protein G HP column (17-0404-01, GE Healthcare). An Isotyping kit was used to determinate the type of immunoglobulin and chain (26179, ThermoScientific): the anti-KYNA antibody was characterized as an isotype Ig G_1 and λ chain. The affinity estimated of the monoclonal anti-KYNA antibody was 10^{-10} M and its specificity was considered excellent because close molecules were not recognized by the antibody (Table 1).

2.3. Immunohistochemical study

Once the tMCAO experimental model was performed (2, 5 or 21 days), the immunocytochemical study was conducted. As previously reported (Mangas et al., 2007, 2012), animals were anaesthetized and perfused and the brains were dissected out, post-fixed and cryoprotected for histological studies. Using a freezing microtome, 40–50 μ-thick brain sections were obtained and processed for immunohistochemistry. In order to avoid possible interference by endogenous peroxidase, sections were treated with methanol and H_2O_2. Later, sections were washed in PBS and pre-incubated in PBS containing Triton X-100 and normal horse serum (mix solution). Sections were incubated overnight at 4 °C in the mix solution containing the monoclonal anti-KYNA antibody (diluted 1/1000, Gemacbio), the monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1/400, Abcam), the polyclonal rabbit anti-GFAP antibody (1/100, Dako) or the polyclonal goat anti-ionized calcium-binding adapter molecule 1 (IBA-1) antibody (1/1500, Abcam). Later, sections were washed in PBS and incubated with biotinylated anti-mouse/rabbit/goat immunogammaglobulin, diluted 1/200 in the mix solution. After a rinse in PBS, sections were incubated with the avidin-biotin-peroxidase complex (ABC) (1/100). Sections were washed in PBS and in Tris–HCl buffer and then the tissue-bound peroxidase was developed with H_2O_2, using 3,3′ diaminobenzidine (DAB) as chromogen. Histological controls were carried out to confirm the specificity of the immunoreactivity: (1) omission of the primary and/or secondary antibodies; and (2) pre-absorption of the anti-KYNA antibody with an excess (100 μg/ml) of KYNA. No residual immunoreactivity was found in either case.

Sections, in which KYNA was detected by the DAB developing procedure, were prepared for double-labelling immunohistochemistry (GFAP: 1/100, Dako) according to a previous published protocol (Marcos et al., 2013). The product of the second immunohistochemical reaction was revealed using 4-chloro-1-naphol as the chromogen. This substance provides a blue precipitate (GFAP) easily distinguishable from the brown product (KYNA) of DAB (Marcos et al., 2013).

The stereotaxic atlas of Paxinos and Watson (1982) was used for mapping and nomenclature. Photomicrographs were obtained with an Olympus DP50 digital camera attached to a Kyowa Unilux-12 microscope. Using a Leica DMRB photomicroscope/Neurolucida system (8.0; Microbrightfield-bioscience, USA) photographs at low-power magnification were also obtained. Adobe Photoshop CS software was used: to improve the visualization of results, only the brightness and contrast of the images were adjusted.

3. Results

Using ELISA tests, the monoclonal anti-KYNA antibody obtained was fully characterized (Table 1). This antibody showed a rather high affinity (the estimated IC_{50} was 10^{-10} M) and an excellent specificity. In these assays, the parameters studied were antibody titration, avidity and specificity versus other close structural analogues (Table 1), according to previously described protocols (Mangas et al., 2007, 2012). ELISA tests were carried out at a dilution of 1/30,000, corresponding to an optical density of 1, at 492 nm (Mangas et al., 2007). Accordingly, competition experiments were performed with different competitors (Table 1). All competition experiments (dilution: 1/30,000) were carried out with the same

Table 1
Affinity and specificity of antibodies against conjugated KYNA.

Compounds	Cross-reactivity at half-displacement (IC_{50})
Kynurenic acid-BSA	1
L-Kynurenine-BSA	1/>50,000
Quinolinic acid-BSA	1/>50,000
Picolinic acid-BSA	1/>50,000
Xanthurenic acid-BSA	1/>50,000
3-Hydroxy-anthranilic acid-BSA	1/>50,000
L-Phenylalanine-BSA	1/>50,000
L-Tryptophan-BSA	1/>50,000

Using competition ELISA tests, cross-reactivity was calculated from the displacement curves at half-displacement: the best recognized conjugate was Kynurenic acid-BSA, whose concentration was divided by the concentration of each of the other conjugates.