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Research article

Increased expression and colocalization of GAP43 and CASP3 after brain ischemic lesion in mouse

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HIGHLIGHTS

- GAP43 and CASP3 are primarily present in neurons after stroke.
- Expression of Gap43 and Casp3 increased after onset of stroke.
- GAP43 and CASP3 colocalized after onset of stroke.

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ABSTRACT

GAP43 is a protein involved in neurite outgrowth during development and axon regeneration reflecting its presynaptic localization in developing neurons. Recently, it has been demonstrated that GAP43 is a ligand of CASP3 involved in receptor endocytosis and is also localized post-synaptically. In this study, by using a transgenic mouse strain carrying a bioluminescent reporter for GAP43 combined with an in vivo bioluminescence assay for CASP3, we demonstrated that one day after brain ischemic lesion and, even more pronounced, four days after stroke, expression of both *CASP3* and *Gap43* in neurons increased more than 40 times. The in vivo approach of CASP3 and GAP43 colocalization imaging was further validated and quantified by immunofluorescence. Importantly, in 82% of GAP43 positive cells, colocalization with CASP3 was present. These findings suggested that one and four days after stroke *CASP3* expression, not necessarily associated with neuronal death, increased and suggested that CASP3 and GAP43 might be part of a common molecular pathway involved in early response to ischemic events occurring after onset of stroke.

1. Introduction

GAP43 (growth-associated protein-43) is a protein associated with neurite growth during development and regeneration of the nervous system. It promotes accumulation of F-actin in neurites, contributing to the formation of the cytoskeleton [1,2]. *Gap43* expression is highest during critical periods of neural system development [3,4]. Perinatally it is expressed in all neurons, but its expression diminishes with decrease of axonal arborization and synaptogenesis and remains only in high-plasticity areas, such as the hippocampus and the olfactory bulb in mice [5]. Recently it has been shown that GAP43 in neurons is a substrate for CASP3 (caspase 3) and that mutation of *Gap43* causes decreased endocytotosis of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor leading to impaired long term depression on the level of postsynaptic membrane [6]. This suggested new roles for both CASP3 and GAP43 in the nervous system.

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After onset of stroke, one of the most prominent regenerative events is axonal sprouting in the penumbra, which is accompanied by high expression of GAP43 [7–10]. Since CASP3 is a protease, not only involved in apoptosis but also in the fine tuning of formation of new synaptic contacts [11], and since GAP43 is a newly discovered postsynaptic substrate for CASP3 [6], we hypothesized that GAP43 and CASP3 together might be part of a recently suggested GAP43/CASP3 common molecular pathway and that it could be involved in tissue response after stroke.

To visualize levels of GAP43 after stroke, we used transgenic mice bearing the luciferase (*luc*) and green fluorescence protein (*gfp*) reporter genes under the control of the murine *Gap43* promoter (C57Bl/6-*Tg(Gap43-luc/gfp) 10Kri*) [12]. By using the luciferase substrate VivoGlo™ Caspase 3/7 Substrate (Promega), we were able to detect a subpopulation of CASP3 positive cells among GAP43 cells.

Our results revealed that expression of both CASP3 and GAP43 after stroke significantly increased (more than 40 times increase by optical density measurements and more than 200 times increase by counting positive cells). Moreover, in 82% of GAP43 positive cells, colocalization with CASP3 was detected. This colocalization further suggests that after stroke CASP3 activity in association with GAP43 could be non-apoptotic.

2. Materials and methods

2.1. Animals

A transgenic C57Bl/6-*Tg(Gap43-luc/gfp) 10Kri* mouse line was generated by Gravel and co-workers and genotyped by polymerase chain reaction (PCR) detection of the luciferase reporter gene as previously described [12,13]. For in vivo imaging 4 animals were used, while ex vivo experiments included 3 groups of animals: baseline ($N=4$), 24 h ($N=5$) and 4 days ($N=5$) with mortality of 5% accounting for 1 animal that reached an ethical endpoint before the experimental time point (2 days) in 4 days group. Total number of animals used was 19.

2.2. Transient middle cerebral artery occlusion (tMCAO)

In order to visualize neuronal responses to injury and repair, we chose the tMCAO method. It is based on unilateral transient focal cerebral ischemia by intraluminal filament occlusion of the left middle cerebral artery for 1 h followed by a reperfusion period previously described [14]. It has been shown that this method gives the highest level of reproducibility and it is the most often used approach in research of brain ischemia/stroke.

2.3. In vivo bioluminescence imaging

As previously described the images were gathered using the IVIS 200 Imaging System (PerkinElmer, Waltham, MA, USA) [13]. The validity of the Gap43 transgenic mice to mirror *Gap43* expression by reporter genes was described previously by one of us [12]. The luciferase substrate VivoGlo™ Caspase 3/7 Substrate (Z-DEVD-Aminoluciferin Sodium Salt, Promega, Madison, WI, USA) was injected intraperitoneally 20 min prior to the imaging session. The mice were anesthetized with 2% isoflurane in 100% oxygen at a flow rate of 2 L/min, placed in the heated light-tight imaging chamber, and maintained anesthetized by constant delivery of the 2% isoflurane–oxygen mixture at 1 L/min through an IVIS anesthesia manifold. To obtain baseline expression measurements, all animals ($N=4$) were imaged before and then first and fourth day following the injury.

The light output was quantified by determining the total number of photons emitted per second (p/s) using the Living Image 4.0 acquisition and imaging software (PerkinElmer, Waltham, MA, USA). Region of interest measurements on the images were used to convert surface radiance (p/s/cm^2/sr) to source flux or total flux of photons expressed in photons/seconds.

2.4. Tissue collection

Animals were anesthetized by an intraperitoneal injection of 2.5% Avertin (Sigma–Aldrich, St. Louis, Mo, USA) and then transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) at pH 7.4 dissolved in phosphate-buffered saline (PBS). Isolated brains were postfixed overnight in 4% PFA, equilibrated in PBS/30% sucrose for 48 h, embedded into Tissue-Tek (O.C.T. compound, Sakura, USA), frozen at −20 °C, cut into 30 μm thick coronal section with a Cryostat (Leica, Wetzlar, Germany), and stored at −20 °C.

2.5. Immunofluorescence

Brain sections were blocked for 30 min in PBS containing 10% goat serum and 0.25% Triton X-100. Sections were then incubated overnight at room temperature with 1:250 primary antibody rabbit polyclonal anti-cleaved CASP3 (Cell Signaling, Danvers, MA, USA), 1:125, 1:250 primary antibody rabbit polyclonal anti-GFP (Merck Millipore, Billerica, MA, USA) used for GAP43 visualization, 1:1000 primary antibody chicken polyclonal anti-GFAP (Abcam, Cambridge, UK) and 1:250 primary antibody mouse monoclonal anti-Neu-N (Merck Millipore, Billerica, MA, USA). Afterwards, sections were incubated for 2 h at room temperature in corresponding secondary Alexa-Flour antibody 1:500 (Invitrogen, Eugene, OR, USA). After both primary and secondary antibody application, slides were given five 10-min washes in PBS with 0.1% Triton X-100 detergent. Two additional washings of 1 min each were done before mounting the slides with Dako Fluorescence Mounting Medium (Dako, Denmark). Before microscopy, slides were left to dry off over night at room temperature.

2.6. Quantification of the immunofluorescent signal

For the quantification of the immunofluorescent signal (Cleaved CASP3, GAP43) four fields of view per section (every sixth section, all ischemic lesion area), ten sections per animal were acquired on a fluorescent microscope (Axiovert 200, Zeiss, Oberkochen, Germany) from the perilesional ipsilateral and corresponding contralateral cortex. Immunoreactivity was quantified with the ImageJ software by measuring the integrated optical density (intensity of fluorescence per unit of surface area), and number of maxima. The results were expressed in arbitrary units as previously described [13]. For the quantification of cells positive for either of markers, the number of positive cells in a field of view was counted manually.

2.7. Confocal imaging and reconstruction

The microscope used was IX81 Olympus microscope equipped with a confocal scan unit FV500 with 3 laser lines: Ar–Kr (488 nm), He–Ne red (646 nm) and He–Ne (532 nm) and UV diode (Olympus, Tokyo, Japan). Images were taken sequentially in Laser Scanning Microscopy (LSM) modality, using a step size as close as possible to pixel size for *z*-axis. Acquired multi-page TIF files were uploaded to Imaris software (Bitplane AG, Zuerich, Switzerland), and processed as described previously [15].

2.8. Statistical analysis

For in vivo imaging results the unpaired Student's *t*-test was used to compare the total photon emission in two different regions