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Ischemia-induced increase in RGS7 mRNA expression in gerbil hippocampus

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Abstract

The present study investigated the changes in the expression of regulators of G-protein-coupled signaling proteins RGS2, 7 and 8 in gerbil hippocampus to better understand alterations of G-protein-coupled receptors signaling after cerebral ischemia. In situ hybridization revealed a transient, robust early increase in RGS7 mRNA levels in the dentate gyrus after ischemia. RGS8 mRNA expression started to increase at a later time point in the CA3 region but no changes were found for RGS2. Our results show a subtype-, time-, and subregion-specific regulation in mRNA expression of RGS proteins after cerebral ischemia in gerbil hippocampus.

Keywords: RGS7; RGS8; RGS2; Cerebral ischemia; Hippocampus

Transient global cerebral ischemia results in selective and delayed neuronal death in distinct hippocampal subregions of the brain [24]. Numerous signaling events have been observed to be activated in cerebral ischemia and in the interplay of these signaling pathways G-protein-coupled receptors (GPCRs) play a pivotal role [11]. It has been reported that a family of proteins called regulators of G-protein signaling (RGS) proteins regulate GPCR functions. Activation of GPCRs catalyzes the exchange of Gα bound GDP for GTP to cause dissociation of Gα from the Gβγ dimer and initiates down stream signaling propagation [13]. RGS proteins are responsible for terminating this signaling by acting as GTPase activating proteins for several G-protein α-subunit members including αt, αq, αo, αi and αz [7]. To date more than 30 mammalian RGS isoforms have been identified, all of which are highly homologous within a ~130 amino acid RGS domain [14]. Although the role of RGS proteins in the central nervous system have not been extensively characterized, recent studies indicate that these proteins play an important role in regulating GPCRs such as, cholinergic, serotoninergic, adrenergic, glutamatergic, GABAergic, etc. receptor functions [14]. An alteration in the function of many of these GPCRs has been observed in cerebral ischemia [11]. Since RGS proteins are known to be selectively regulated at the level of gene expression, we have investigated their dynamic regulation in response to an ischemic insult. In particular, we examined the expression pattern and distribution of RGS2, -7 and -8 subtypes in hippocampal subregions of gerbil brain at different time points after ischemia.

Adult male Mongolian gerbils (60–80 g body weight; Charles River, Wilmington, MA) were fed and housed as previously described [34,35]. Animals were randomly divided into three groups: sham controls ($n = 6$), 4 h after ischemia ($n = 7$), and 16 h after ischemia ($n = 8$) for in situ hybridization studies, and sham controls ($n = 7$), 8 h after ischemia ($n = 7$) and 16 h after ischemia ($n = 8$) for Western blot analysis. Surgery procedures were the same as described in our earlier reports [34,35]. Briefly, transient global cerebral ischemia was induced by occlusion of both common carotid arteries for 5 min under anesthesia with isoflurane (2.5%), nitrous oxide (70%), and oxygen (30%). Regional cerebral blood flow (rCBF) in both sides of the forebrain was measured by a laser Doppler flowmeter (MBF3D, Moor Instruments, Devon, UK). Gerbils showing a decrease in rCBF of less than 80% were excluded from subsequent analyses. Sham operated animals underwent the same procedure except for the occlusion of the carotid arteries [34,35]. Experiments were performed in strict compliance with NIH guidelines. The protocol

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of this study was reviewed and approved by the University of Missouri-Columbia Animal Care and Use Committee.

In situ hybridization was carried out as previously described [29]. Briefly, 12 μm coronal sections were fixed in 4% paraformaldehyde/phosphate-buffered saline for 5 min. After treatment with acetic anhydride triethanolamine hydrochloride, slides were dehydrated through a graded series of ethanol, delipidated in chloroform, rehydrated, and air-dried. Fifty microliters of hybridization buffer containing 1×10^6 cpm ^{35}S-radiolabeled oligonucleotide probe was applied to each slide, covered with a parafilm coverslip, and incubated at 42 °C overnight. After hybridization, slides were washed in 1× SSC (150 mM NaCl/15 mM sodium citrate) at 55 °C for 4 × 15 min. Following two 30 min rinses in 1× SSC at room temperature, the tissues were dipped in distilled water, immersed in 70% ethanol, and air-dried.

Oligonucleotides were 3′ end-labeled by terminal deoxynucleotidyl transferase (Roche, Indianapolis, IN) with ^{35}S-dATP (NEN, Boston, MA). The probes for the RGS subtypes were the same as used by Grafstein-Dunn et al. [9] and Gold et al. [8].

Slides were held against KODAK BIOMAX MR films with standards (American Radiolabeled Chemicals Inc., St. Louis, MO, USA) in X-ray cassettes. Microdensitometry of film autoradiograms was performed on the signal over different hippocampal subregions using the BIOQUANT True Color Windows 95 software version 2.50 as previously described [29,30]. [^{14}C]-microscale standards were used to construct calibration curves and quantitate signals in μCi/g tissue equivalents. The average density measured from experimental regions fell within the linear range of the standards. Background signal was subtracted from all measurements. Resulting values were averaged from four sections for each animal before being evaluated for statistical significance.

Western blot analysis was performed as described by Khawaja et al. [17]. Briefly, hippocampi were dissected and homogenized in homogenization buffer (Tris-HCl, EDTA, sucrose, pH 7.4, benzamide, iodoacetamide and PMSF). The homogenate was centrifuged at 6000 rpm at 4 °C for 10 min and the clear supernatant was stored at −80 °C until further use. Protein concentrations were determined by the Bradford assay [3], with bovine serum albumin as the standard. Equivalent amounts of protein (40 μg) for each sample were resolved in 10% SDS-PAGE in duplicates. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated in Tris-buffered saline, pH 7.4 (TBS) with 0.5% Tween 20 (TBS-T) containing 5% nonfat milk overnight at 4 °C. The blots were reacted with rabbit anti-RGS7 (1:500; Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature. After washing with TBS-T, they were incubated with goat anti-rabbit IgG-HRP (1:2000; Sigma, St. Louis, MO) for 1 h at room temperature. Then the blots were washed three times with TBS-T. Immunolabeling was then detected by chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL). The blots were striped using the standard protocol, washed and re-probed with mouse anti-β-actin (1:1000; Cytoskeleton, Denver, CO). For quantification, blots were scanned with a scanner and intensity of protein bands was measured as optical density using the Quantity One program (BioRad, Hercules, CA). Initially, optical densities of increasing amount of protein (10–80 μg) were measured and a linear relation between protein concentration and optical density was determined. RGS7 bands were detected at 55 kDa and β-actin bands were detected at 43 kDa. Ratios of RGS7 to β-actin were calculated for each sample.

Data were expressed as mean ± S.E.M. and analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test (V4.00, GraphPad Prism Software, Inc., San Diego, CA). A value of $p < 0.05$ was accepted as significant.

Initially, rat and gerbil coronal sections were ran in parallel and probed for RGS2, 7 and 8 mRNAs (data not shown). We found the distribution pattern of all the three subtypes to be similar for gerbil and rat brain and also identical to pervious studies [8,16]. Table 1 shows the changes in mRNA expression of RGS subtypes in hippocampal subregions after cerebral ischemia. The expression of RGS2 mRNA in all the subregions remained unaltered both at 4 and 16 h after ischemia. However, RGS7 and RGS8 mRNA levels were elevated following ischemia. There was an increase in RGS7 mRNA by 50–60% in the dentate gyrus at 4 h after reperfusion (Fig. 1). Then, the mRNA levels decreased between 4 and 16 h but values were still significantly higher than controls. RGS8 mRNA showed an increase

Table 1
Changes in the mRNA expression of RGS2, RGS7 and RGS8 in gerbil hippocampal subregions after ischemia

Regions	RGS2			RGS7			RGS8		
	4 h	16 h	*p*-values	4 h	16 h	*p*-values	4 h	16 h	*p*-values
CA1	95.1 ± 5.2	110.0 ± 9.1	0.3161	116.2 ± 8.1	111.2 ± 9.3	0.3370	97.1 ± 9.1	126.1 ± 10.3	0.1099
CA3	97.8 ± 4.4	107.1 ± 6.7	0.3822	108.1 ± 7.5	105.3 ± 7.5	0.7363	103.8 ± 6.2	139.7 ± 9.4[b]	0.0041
ObDG	97.0 ± 6.3	110.3 ± 9.4	0.4319	159.7 ± 10.1[a]	127.8 ± 4.8[c,d]	<0.0001	91.1 ± 9.8	122.9 ± 9.3	0.0840
IbDG	86.1 ± 6.5	102.9 ± 8.6	0.2505	150.5 ± 8.6[a]	122.9 ± 4.6[c,d]	0.0002	92.1 ± 6.4	97.6 ± 12.2	0.8760
PoDG	97.1 ± 6.1	106.5 ± 9.1	0.6030	114.8 ± 7.5	97.9 ± 3.2	0.3156	100.0 ± 10.5	117.1 ± 9.8	0.4078

Data are expressed as percent of the corresponding values found in sham controls (mean ± S.E.M. from 6–8 animals per group). Data are analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test. ObDG, outer blade of dentate gyrus; IbDG, inner blade of dentate gyrus; PoDG, polymorph layer of dentate gyrus.

[a] $p < 0.001$ between sham and ischemia.
[b] $p < 0.01$ between sham and ischemia.
[c] $p < 0.05$ between sham and ischemia.
[d] $p < 0.01$ between 4 and 16 h after ischemia.