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Neuroscience Letters 256 (1998) 65–68

Neuroscience Letters

Simultaneous assay of ornithine decarboxylase and polyamines after central nervous system injury in gerbil and rat

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Received 19 May 1998; received in revised form 2 September 1998; accepted 11 September 1998

Abstract

Ornithine decarboxylase (ODC) is considered the rate-limiting enzyme in polyamine biosynthesis. An increase in putrescine (a natural polyamine) synthesis after central nervous system (CNS) injury appears to be involved in blood-brain barrier dysfunction, development of vasogenic edema and neuronal death. An improved method is described to determine the ODC activity as well as polyamine levels from the same brain tissue. The polyamine results showed no significant differences from data obtained with the conventional assay. The advantages of this method are to: (1) minimize the number of animals needed for the study, and (2) eliminate any internal inconsistencies resulting from use of two independent groups of animals for ODC and polyamine measurements. Using this method, ODC activities and polyamine levels were measured in cortices and hippocampi from global transient ischemia of gerbils and traumatic brain injury (TBI) of rats.

Keywords: Cerebral ischemia; Cortex; Hippocampus; Ornithine decarboxylase; Polyamines; Putrescine; Traumatic brain injury

The polyamines are a class of ornithine-derived molecules which play a role in cell proliferation, differentiation, growth, calcium mobilization, and membrane transport functions [12,13,22]. Ornithine decarboxylase (ODC) converts ornithine to putrescine and is the rate-limiting step in polyamine biosynthesis [22]. ODC activity is induced by several central nervous system (CNS) injuries including cerebral ischemia and traumatic brain injury (TBI) [1,3,5,8,16]. Induction of ODC activity is a delayed response to CNS injury involving ODC gene expression at 1–4 h post-injury [6,11] and a substantial increase in ODC protein synthesis at 6 h [19].

Previous work from our laboratory has shown induction of ODC and subsequent alterations in polyamine metabolism, particularly an increase in putrescine levels, to be an important factor in blood-brain barrier dysfunction and development of vasogenic edema after CNS injury [1,2,16,20]. The post-injury increase in putrescine may alter membrane calcium fluxes [12] and has been hypothesized to play a role in the delayed neuronal death after CNS injury [8,11,14,15,21].

Earlier studies examining ODC activity and polyamine levels used two independent groups of animals to determine these two endpoints. We describe here an improved method in which both ODC activity and polyamines are measured from the same brain tissue. The main advantages of this method are to: (1) reduce by one-half the number of animals required for the studies, and (2) eliminate any internal inconsistencies resulting from use of two independent groups of animals for ODC and polyamine measurements. This present study applied this method to examine the regional activities of ODC and polyamines in: (1) transient ischemia of gerbils and (2) TBI of rats. The polyamine results were compared between simultaneous assay (polyamines extracted from tissue homogenate in ODC buffer by perchloric acid) versus conventional assay (polyamines

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0304-3940/98/$19.00
PII S0304-3940(98)00780-0

extracted by homogenizing the tissue in perchloric acid). To the best of our knowledge this is the first report simultaneously measuring the ODC activity and its product, putrescine from the same cerebral tissue after CNS injury.

Tris–HCl, pyridoxal-5-phosphate, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), 1,7-diaminoheptane, and dansyl chloride were obtained from Sigma (St. Louis, MO, USA). L-[1-^{14}C]ornithine was obtained from New England Nuclear Research Products (Boston, MA, USA).

All surgical procedures were conducted according to the animal welfare guidelines set forth in the NIH Guide for the care and use of laboratory animals (US Department of Health and Human Services, Publication 85-23, 1985) and were approved by the animal care committee of the University of Wisconsin-Madison. Male Mongolian gerbils (50–80 g) common carotid arteries were occluded for 10 min under halothane anesthesia and reperfused for 6 h as described [6,16]. Control animals underwent the same surgical procedure except the vessels were not occluded. Body temperature was maintained at 37–38°C during ischemia and reperfusion.

Male Sprague–Dawley rats (300–350 g) were anesthetized with 1.5% halothane in a 50:50 mixture of N_2O:O_2. TBI was induced by using a controlled cortical impact device described earlier [1,2,18,19]. Each rat in the experimental groups was injured with a 6-mm diameter tip at a velocity of 3 m/s and 2 mm deformation while rats in the control group were subjected to the same surgical procedure, but received no impact. Mean arterial blood pressure and arterial blood gas levels were monitored and maintained within physiological limits. Core and cranial temperatures were monitored with rectal and temporalis muscle probes and maintained at 37–38°C for rectal and at 36–37°C for temporalis muscle with a heating pad and a lamp during experiments.

Animals were sacrificed, cortex and hippocampus from both hemispheres were dissected at 4°C and immediately frozen in liquid N_2. The brain regions were homogenized in ODC assay buffer (50 mM Tris–HCl, pH 7.8, 40 μM pyridoxal-5-phosphate, 7.5 mM DTT, 1.0 mM EDTA, and 500 μM PMSF) at 4°C. The homogenates were then divided into two equal aliquots.

One aliquot of the tissue homogenate was centrifuged at 16 000 × g for 20 min. ODC activity in the cytosol fraction was measured by determining the amount of $^{14}CO_2$ released from 0.5 μCi of L-[1-^{14}C]ornithine at 37°C during a 1 h incubation by a method described previously [1,16]. The cytosol was assayed for Lowry protein. ODC activity was expressed as pmol/mg cytosolic protein per h.

In the simultaneous assay, to the second aliquot of the tissue homogenate, 9.2 N perchloric acid was added to a final concentration of 0.2 N. For the conventional polyamine assay, a different set of animals underwent similar CNS injury (cerebral ischemia/reperfusion and TBI) and the brain regions (cortices and hippocampi) were isolated. The brain tissue was homogenized in 0.2 N perchloric acid. Homogenates from simultaneous and conventional assays were centrifuged at 16 000 × g for 20 min. After removal of the supernatant, the pellets were redissolved in 1 N sodium hydroxide and assayed for total Lowry protein.

Polyamines in the supernatant were determined as described earlier [3,10] with minor modifications. The supernatants were dansylated, applied to Bond-Elut C-18 columns (Varian Associates, Harbor City, CA), and the dansylated polyamines were eluted with 1.5 ml of acetonitrile. Samples were diluted with an equal volume of 90% 10 mM sodium acetate, pH 4.5–10% acetonitrile (Solvent A) and 50 μl were injected into a Hewlett-Packard 1100 high-performance liquid chromatograph (HPLC). The instrument was fitted with a Waters 8 × 100 mm Nova-Pak column and radial compression module. The column was eluted with a mixture of 48% acetonitrile and 52% Solvent A at a flow rate of 3 ml/min with a linear gradient to 90% acetonitrile from 0 to 25 min, and 90–100% acetonitrile from 25 to 30 min. The polyamines were quantitated against internal (1,7-diaminoheptane) and external standards using a Hewlett-Packard 1046A fluorescence detector with λ_{ex} 340 nm and λ_{em} 515 nm. Polyamine levels were expressed as nmol/mg total protein. All the measurements were expressed as mean ± SD. Data were analyzed using a one-way ANOVA with the Bonferroni test to compare differences between the groups (GraphPad Software, San Diego, CA). A value of $P < 0.05$ was considered significant.

On HPLC analysis no interfering peaks were observed in the chromatogram obtained from dansylated ODC assay buffer. No difference in polyamine recovery was observed when the tissue was homogenized in ODC assay buffer followed by perchloric acid extraction (simultaneous assay) versus single step extraction (conventional assay) with perchloric acid.

In previous studies, the average putrescine levels in one group of animals could be compared with ODC activity in a parallel group. The current method allows the data for both parameters to be obtained from the same tissue, and individual variations in ODC activity can be shown with respect to the putrescine levels. A direct comparison between the conventional (extracting the tissue polyamines by perchloric acid treatment) and the simultaneous methods (extracting the polyamines from tissue homogenate in ODC buffer by addition of perchloric acid) did not show any significant differences in putrescine levels (Table 1). Spermidine and spermine levels also did not show any significant differences between these two methods (data not presented).

Table 1 summarizes the ODC activities and putrescine levels after transient ischemia in gerbils and TBI in rats. In ischemic gerbils, both ODC activity and putrescine levels in cortex and hippocampus were significantly elevated compared to respective regions of shams ($P < 0.001$).

Putrescine levels versus ODC activity in individual rats 6 h after TBI are summarized in Table 1. In TBI rats, both ODC activity and putrescine levels were elevated in ipsilat-

Table 1
ODC activities and putrescine levels after transient ischemia in gerbils and TBI in rats

Transient ischemia in gerbils				Traumatic brain injury in rats						
Region	6 h reperfusion			Region	6 h after injury			24 h after injury		
	Simultaneous[1]		Conventional[2]		Simultaneous		Conventional	Simultaneous		Conventional
	ODC[a]	Putrescine[b]	Putrescine[b]		ODC[a]	Putrescine[b]	Putrescine[b]	ODC[a]	Putrescine[b]	Putrescine[b]
Sham C	5.3 ± 1.0	0.056 ± 0.001	0.052 ± 0.001	Sham C	5.1 ± 1.0	0.024 ± 0.009	0.022 ± 0.01	5.1 ± 1.0	0.024 ± 0.009	0.021 ± 0.01
Sham H	6.0 ± 1.0	0.057 ± 0.005	0.059 ± 0.007	Sham H	4.8 ± 0.8	0.027 ± 0.013	0.025 ± 0.015	4.8 ± 0.8	0.027 ± 0.013	0.028 ± 0.011
Isch. C	36.1 ± 1.9*	0.13 ± 0.01*	0.125 ± 0.095*	IC	89.6 ± 4.3**	0.393 ± 0.024**	0.41 ± 0.027**	14.9 ± 0.7**	0.969 ± 0.110**	0.982 ± 0.098**
Isch. H	37.0 ± 2.0*	0.13 ± 0.01*	0.121 ± 0.009*	IH	87.0 ± 4.5**	0.414 ± 0.039**	0.39 ± 0.042**	18.6 ± 1.1**	0.963 ± 0.102**	0.951 ± 0.10**
				CC	8.4 ± 3.3	0.053 ± 0.023	0.049 ± 0.025	3.6 ± 0.9	0.096 ± 0.038	0.092 ± 0.036
				CH	9.3 ± 2.5	0.054 ± 0.017	0.052 ± 0.016	4.4 ± 1.2	0.094 ± 0.048	0.094 ± 0.05

[1]Simultaneous (tissue was homogenized in ODC assay buffer and polyamines were extracted from an aliquot by addition of perchloric acid). [2]Conventional (separate set of animal tissue was used to extract polyamines using perchloric acid). Data are the mean ± SD. [a]pmol/mg cytosolic protein/h; [b]nmol/mg total protein. Isch., Ischemia; C, Cortex; H, Hippocampus; *$P < 0.001$ compared to respective sham regions. Sham, $n = 3–4$ and ischemic, $n = 5–7$ per group. IC, ispsilateral cortex; IH, ipsilateral hippocampus; CC, contralateral cortex; CH, contralateral hippocampus. **$P < 0.001$ compared to respective sham and contralateral regions. Sham, $n = 3–4$ and TBI, $n = 6–7$ per group.

eral cortex (IC) and hippocampus (IH) compared to contralateral cortex (CC) and hippocampus (CH) respectively and shams ($P < 0.001$). There was no statistically significant difference between CC and CH compared to shams.

At 24 h after TBI the putrescine levels continued to increase in the IC and IH. The increases in putrescine levels in the IC and IH were significant when compared to IC and IH levels at 6 h ($P < 0.001$) or with the respective contralateral regions or shams ($P < 0.001$). The ODC activities in IC and IH significantly decreased from 6 h ($P < 0.001$), but were still significantly higher than in the contralateral regions and shams ($P < 0.001$). No significant differences in ODC activity or putrescine levels were observed in CC and CH compared to the respective sham regions at 6 or 24 h. Spermidine and spermine levels were not significantly altered in either gerbil ischemia/reperfusion or in rat TBI compared to their respective controls (data not shown).

In these studies, an increase in ODC activity correlated with an increase in putrescine levels in gerbil ischemia/reperfusion and in rat TBI determined at 6 h after the CNS injury. Our previous studies have shown that ODC mRNA is expressed from 1–4 h after transient ischemia in gerbils [6,11], and ODC protein levels as well as enzyme activity in rat TBI were elevated at 6 h after the injury [1,16].

At 24 h after TBI, the ODC activity and protein levels were approaching contralateral levels [19] but still remained elevated (Table 1). In contrast, putrescine levels continued to increase at 24 h in the ipsilateral regions (Table 1). This finding is in agreement with Paschen et al. [14,15], where the putrescine levels after global ischemia/reperfusion showed a similar trend suggesting that transient ischemia and TBI may share common polyamine metabolic pathways. However, Henley et al. [8] showed an increase in both ODC activity and putrescine levels at 8 h after TBI, but did not observe putrescine levels increase from 8 to 24 h.

The cerebral ODC/polyamine system is disturbed by CNS injury. The main modifications are significant increases in ODC activity and putrescine concentration, with minor or no variations in spermidine and spermine levels [5,14]. In recent years it has been found that the ODC/polyamine system is very sensitive to different cerebral pathological states [5,14]. Alterations in polyamine metabolism correlate with the degree of injury and with neuronal death in the CA_1 region of the hippocampus [8,11,12,14,17,20]. While ODC is considered the rate-limiting enzyme in polyamine biosynthesis [22], it is not the sole pathway accounting for putrescine levels in tissues. Activities of *S*-adenosylmethionine decarboxylase, the enzyme that provides the aminopropyl groups necessary for the conversion of putrescine to spermidine and spermine, has been shown to decrease after CNS injury [7,9,15,17]. At 24 h after CNS insult, putrescine accumulates even while ODC activity is declining to near basal levels. This suggests that activation of interconversion pathway enzymes, spermidine/spermine N^1-acetyltransferase [23] and polyamine oxidase [4], which convert spermine to spermidine and spermidine to putrescine, probably is a major factor in putrescine accumulation at 24 h. However, the role of polyamines, especially putrescine, in the pathology of CNS trauma and ischemia is not clear and needs to be further investigated.

In summary, we have described here a modification to previous methods which will allow measurement of ODC and polyamines from the same brain tissue. By dividing the

tissue homogenate, uniform samples are assured for both measurements. This avoids inconsistencies which might arise from assaying tissue sections (e.g. punch biopsies), particularly where the injury is non-uniform (such as focal ischemia or mechanical trauma) or the use of two independent groups of animals.

This study was supported by start-up funding from University of Wisconsin (to A.M.R.) and grants from NIH (RO1 NS 28000, PO1 NS31220), and Department of Veterans Affairs (to R.J.D).

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