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Research report

Regional brain polyamine levels in permanent focal cerebral ischemia

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Abstract

Transient global cerebral ischemia has been shown to induce marked changes in the polyamine pathway with a significant increase in putrescine, the product of the ornithine decarboxylase reaction. This study examined the relationship between tissue and extracellular polyamines and regional cerebral blood flow and brain edema. Six hours of focal ischemia in cats ($n = 10$) was produced by permanent middle cerebral artery occlusion. Extracellular polyamines were measured in extracellular fluid obtained by microdialysis. Regional cerebral blood flow using laser Doppler flowmetry and specific gravity, an indicator of brain edema, were measured in contralateral (non-ischemic), penumbra and densely ischemic brain regions. A significant increase in the tissue putrescine level was found in the penumbra but there was no difference in the putrescine levels between contralateral and densely ischemic regions. There was no significant change in the spermidine and spermine levels in the three regions. Extracellular levels of putrescine and spermidine were found to be significantly lower than the tissue levels and no change in polyamines was observed in any region. Significant edema formation was observed in densely ischemic and penumbra regions. This is the first demonstration that tissue putrescine is increased in the penumbra region, an area of incomplete ischemia that is developing brain edema.

Keywords: Brain edema; Cerebral blood flow; Focal cerebral ischemia; Polyamine; Putrescine

1. Introduction

Polyamines are a class of ornithine-derived molecules noted for their role in cell growth, cell differentiation, membrane transport functions, protein synthesis, and calcium mobilization [22,23,31]. It is known that the ornithine decarboxylase (ODC) derived polyamine pathway is provoked by several types of brain injury including cerebral ischemia [9,29,32], traumatic brain injury [1] and cryogenic injury [24]. Among them, cerebral ischemic injury has several subtypes regarding the localization and reversibility of ischemia. Polyamine metabolism is widely studied in transient global ischemia and it has been shown that the change in metabolism of the ODC polyamine pathway consists of a transient elevation of ODC activity, peaking at 4–8 h of reperfusion. ODC polyamine pathway has also been shown to be involved in the production of cerebral edema following ischemia and reperfusion injury [32] and to function as modulators of both calcium fluxes at the cell membrane and neurotransmitter release from nerve endings [4]. In transient global ischemia models measurement has been made almost exclusively of tissue polyamines. An exception is the study of Gilad et al. [17] of extracellular polyamines in microdialysates in rats subjected to transient global ischemia. However, in that study they did not find any change in extracellular polyamines detected for up to 3 h of reperfusion.

In contrast to reversible transient ischemia, information on polyamine metabolism in focal cerebral ischemia is limited and polyamine metabolism has been measured primarily in brain tissue. In this study we examined this metabolism in a model of focal cerebral ischemia. The cat model of focal cerebral ischemia produces a focal flow deficit sufficient to cause a well-defined cerebral infarct (densely ischemic) in the inferior and lateral hemispheres, specifically the inferior portion of the anterior and posterior ectosylvian regions. In this densely ischemic region severe reductions of cerebral blood flow (CBF) (< 10–12 ml/100 g/min) are seen [7,8]. The dense area of ischemia is then surrounded by an area of moderate ischemia in

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which reductions of CBF are damaging but may not be immediately lethal. In this region cells are still viable but may be electrically silent (penumbral region, 18–20 ml/100 g/min) [7,8,35]. Therefore, the present study examined extracellular polyamines in the penumbral and contralateral (non-ischemic) brain regions and tissue polyamines in the contralateral (non-ischemic), penumbral and densely ischemic regions in cats subjected to 6 h of permanent focal ischemia.

2. Materials and methods

In this study, we carefully adhered to the animal welfare guidelines set forth in the *Guide for the Care and Use of Laboratory Animals*, US Department of Health and Human Services, Publ. No. 85-23, 1985.

2.1. Preparation of animals

Ten healthy mongrel cats of either sex weighing 2.5 to 4.5 kg were fasted with free access to water for approximately 24 h prior to the experiments. Cats were anesthetized with pentobarbital (25 mg/kg i.v. followed by 4 mg/kg/h). The cats were intubated and respiration was controlled by a respirator to maintain arterial blood gas concentrations and pH within normal physiological limits. Femoral venous and arterial catheters were placed for slow continuous saline infusion and monitoring of the mean arterial blood pressure and heart rate. Blood gases and hematocrits were obtained during surgical procedures just before middle cerebral artery (MCA) occlusion, at 1, 3, and 5 h after occlusion, and at the end of each experiment. Rectal temperature was measured continuously with a telethermometer (YSI 2100, Yellow Springs Instrument Co., Yellow Springs, OH) and was maintained between 37°C and 38°C by a water blanket (American Hospital Supply Corp., Valencia, CA).

2.2. Focal ischemia model

In the cat, occlusion of the right middle cerebral artery (MCA) was performed just distal to the circle of Willis by a transorbital technique as described previously [7,8]. Briefly, after complete exenteration of the orbit, the optic foramen and optical fissure were enlarged with a high speed drill cooled by saline. The dura was incised and then the MCA was exposed. The MCA was dissected free from the arachnoid membrane and a 5-0 prolene suture was looped around the artery. After the surgical procedure was completed, the animals were placed in a stereotaxic device for cats and continuously monitored for CBF. After baseline values were obtained, each end of the prolene was passed through a double-lumened vinyl tube (3 cm long, 1.1 mm i.d., 2.5 mm o.d.). Occlusion of the MCA was obtained by applying traction to the prolene. Traction was maintained with two aneurysm clips. Ischemia was confirmed by the decreased CBF as measured by a laser flow meter. The anesthetized animals were humanely put to death with saturated KCl solution at the end of 6 h of occlusion time, and a complete craniectomy was performed, exposing the brain down to cervical laminae-1.

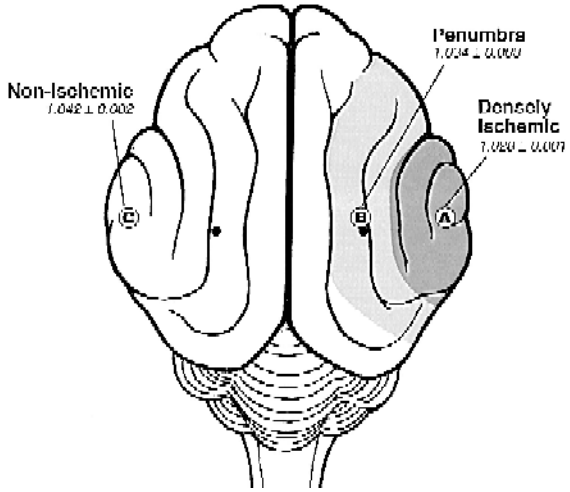

Fig. 1. Illustration of the cerebral blood flow measurements and regional distribution of ischemic brain edema. Sampling of brain tissue for specific gravity and tissue polyamine measurements was performed from the cortex adjacent to each laser flowmetry probe of densely ischemic (A), penumbra (B), and contralateral non-ischemic (C) regions. Microdialysis probes were placed into the right parietal cortex (penumbra) near the point B and left parietal cortex (nonischemic hemisphere). Both were 10 mm lateral to midline (black circles). Values are expressed as the mean ± S.E.M.

2.3. Regional cerebral blood flow measurement

For CBF measurement, we used a laser Doppler flowmetry (LDF) monitor equipped with a small-caliber probe of 2-mm diameter (Laserflo Blood Perfusion Monitor, BPM 403A, TSI, MN). The laser Doppler flowmetry provides instantaneous, continuous, and noninvasive measurements of microcirculatory blood flow in a tissue sample of ~ 1 mm^3 [10]. It has been shown that there is a good correlation between LDF and other techniques of monitoring blood flow in the brain microcirculation [17,34]. The scalp was incised in the midline and reflected. Flow was measured at three points on the surface of the cortex. Point A was placed 22 mm lateral to the midline, point B, 10 mm lateral to the midline in the ipsilateral ischemic hemisphere, and point C, 22 mm lateral to the midline in the contralateral hemisphere (Fig. 1). We have previously shown that in this model point A marks a well-defined cerebral infarct in a core (densely ischemic) region with severe reductions of CBF (10–12 ml/100 g/min). Point B marks a penumbral region in which the reduction of CBF is damaging but not lethal (18–20 ml/100 g/min [7,8]. Point C is contralateral to the ischemic site. At each point, an 4-mm hole was drilled through the skull, leaving the

dura intact. Large blood vessels were avoided by microscopic guidance. The LDF probe was held in a micromanipulator and advanced to gently touch the dura mater. To prevent desiccation of the exposed brain, warmed 9% saline solution was slowly rinsed around the probe during the experiment. Stable baseline LDF readings were obtained for at least 30 min from all sites of the MCA before occlusion. A continuous digital display of LDF values was averaged over 5-s intervals and recorded just before occlusion, 30 min after occlusion, and then every hour during the MCA occlusion. The CBF values were calculated and expressed as a percentage of the baseline values.

2.4. Procedure for microdialysis

Microdialysis probes (CMA/12, membrane length 3 mm, diameter 0.5 mm; 2701 West Lafayette, IN 47906) were perfused continuously with Ringer solution (NaCl 147.1 mM, KCl 4.0 mM, $CaCl_2$ 2.25 mM) at a constant rate of 2.0 μl/min using a micro-infusion pump (Model A-99, Razel Scientific Instruments, Stanford, CT). Two 2-mm holes were drilled through the skull over the penumbral region and the contralateral hemisphere. Microdialysis probes were placed 3 mm into the right parietal cortex (penumbra) and left parietal cortex (contralateral, nonischemic) with a stereotaxic micro-manipulator (David Kopf Instruments, Tujunga, CA) and secured with dental impression material (Kerr Polysulfide Impression Material, Type 2, Kerr Manufacturing Co., Romulus, MI) (Fig. 1). Dialysate was collected every hour in a fresh eppendorf tube containing 0.2 N $HClO_4$ placed on ice. It has been shown that extracellular concentrations of metabolites stabilize within 1 h after the insertion of the probe [3]. Therefore, the probes were perfused for 1 h before MCA occlusion, after which the occlusion and microdialysis were continued for 6 h.

2.5. Measurement of brain polyamines

After the animals were humanely put to death, the brain was quickly removed and $\sim$2-mm^3 samples of tissue from the cortex adjacent to each laser flowmetry probe of densely ischemic, penumbral, and contralateral regions were taken for tissue polyamine measurements. These samples were immediately frozen in liquid N_2 and stored at -70°C until assay. Polyamine content was evaluated as described by Kabra et al. [20]. The frozen brain tissue samples were homogenized in 5 volumes of 0.2 N $HClO_4$. The homogenates were then centrifuged at $16\,800 \times g$ for 20 min. The supernatants were used for dansylation. The supernatants and dialysate were dansylated, applied to a Bond-Elut C18 column, and the dansylated polyamines were eluted with 1.5 ml methanol. Samples (10–50 μl) were then injected onto a 4 μM Waters Nova-Pak C-18 HPLC column (3.9 mm $\times$ 75 cm) and polyamines were quantitated with a Waters HPLC 501 system equipped with a model 470 spectrofluorometer. The limit of detection for putrescine, spermidine, and spermine is 1 pmol. Polyamine contents were normalized to the weight of the tissue sample/g or /ml of densylate.

2.6. Cerebral edema

Brain edema was assessed by determining regional specific gravity as an indicator of regional water content [1,2,28,32]. Tissue samples from each of three brain regions to be studied (as described in tissue polyamine measurement) were quickly removed in 1-mm^3 pieces and placed in kerosene. A kerosene-bromobenzene continuous gradient column was used for testing of specific gravities. The column was calibrated with K_2SO_4 samples of known specific gravity. Each tissue sample was placed at the top of the column and allowed to equilibrate for 1 min. The distance from the sample to the bottom of the column was measured to determine its specific gravity.

2.7. Statistical analysis

All measurements were expressed as the mean $\pm$ S.E.M. One-factor Anova with Bonferroni test was used for comparing between two groups using Graph Pad Instat software (ISI software, San Diego, CA). Results were considered significant when $P < 0.05$.

3. Results

3.1. Physiological parameters

No significant changes were observed in blood pressure, heart rate and arterial blood gases during the experiments.

3.2. Regional cerebral blood flow

Occlusion of the MCA produced a significant fall in cortical blood flow at point A (dense ischemic) and point B (penumbra) ($P < 0.05$). CBF was reduced to 6 ± 1% of base-line at point A and 35 ± 4% at point B at 1 h after occlusion of the MCA and remained 7 ± 2% of base-line at point A and 28 ± 5% point B at the end of the occlusion. At point C (contralateral region), 1 h after occlusion of the MCA, the mean percentage of baseline CBF was 102 ± 3% and remained 92 ± 4% at the end of the occlusion period.

3.3. Tissue polyamine levels

The densely ischemic and contralateral regions showed similar putrescine levels (Fig. 2). A twofold increase in putrescine levels was found in the penumbral region ($P < 0.05$) (Fig. 2). There was no significant change in the

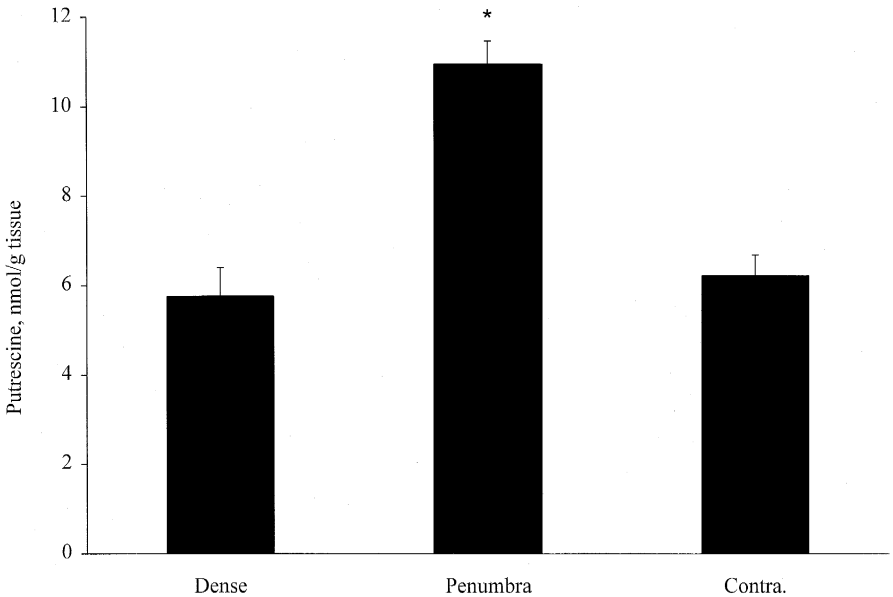

Fig. 2. Tissue putrescine levels in the densely ischemic, penumbra and contralateral brain regions in cats subjected to 6 h of middle cerebral artery occlusion. Values are expressed as the mean ± S.E.M. * $P < 0.05$.

spermidine and spermine levels in the three regions. Spermidine levels were 88.3 ± 9.4 in the densely ischemic, 112.8 ± 18.7 in the penumbra, and 123.6 ± 17.8 in the contralateral regions. Spermine levels were 59.6 ± 6.2 in the densely ischemic, 58.4 ± 5.5 in the penumbra, and 64.1 ± 4.5 in the contralateral regions.

3.4. Extracellular polyamine levels

Extracellular polyamine levels were measured in the penumbra and contralateral brain regions. Extracellular levels of polyamines were found to be significantly lower than the tissue levels. There was no significant change in the extracellular putrescine levels during the 6 h MCA occlusion (Fig. 3). Extracellular putrescine levels were 0.20 ± 0.07 in the penumbra and 0.29 ± 0.01 in the contralateral regions before occlusion and were 0.54 ± 0.5 in the penumbra and 0.25 ± 0.07 in the contralateral regions at 1 h after occlusion. These levels remained unchanged significantly throughout the experiments (0.33 ± 0.2 in the penumbra and 0.27 ± 0.05 in the contralateral regions at 6 h after occlusion). Extracellular spermidine levels were

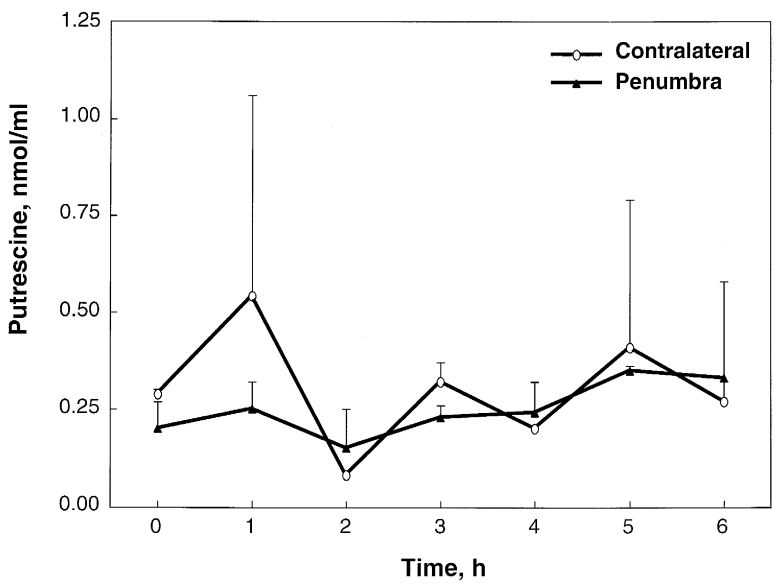

Fig. 3. Extracellular putrescine levels in the penumbra and contralateral brain regions in cats subjected to 6 h of middle cerebral artery occlusion. Values are expressed as the mean ± S.E.M.

very low and did not show any significant change throughout the experiments (data not shown). Extracellular spermine was not detected in any brain region.

3.5. Cerebral edema

Specific gravity values of densely ischemic, penumbra, and contralateral regions were 1.028 ± 0.001, 1.034 ± 0.003, and 1.042 ± 0.002. Six hours of focal cerebral ischemia produced significant edema in the dense ischemic and penumbral regions (Fig. 1). Increased cerebral edema is indicated by decreased specific gravity.

4. Discussion

The results of this study revealed that tissue level of putrescine increased in the penumbra region but spermidine and spermine levels did not change significantly either in penumbral or densely ischemic regions. Although these findings are consistent with previous reports in transient global ischemia there are some differences which should be pointed out. First, the increase in tissue putrescine level in permanent focal ischemia is less prominent than that of transient global or transient focal ischemia. Paschen et al. [30] have found tissue putrescine levels of 47 nmol/g in the cortex and 53.6 nmol/g in the striatum after transient focal ischemia in rats, even these levels are only 35% of that found after transient global ischemia. Second, this increase is significant only in the penumbra region, the region of incomplete ischemia, suggesting that for induction of the polyamine pathway continuation of blood flow at least at penumbral levels or eventual recirculation is necessary. Moreover, it is known that cellular polyamine synthesis is also controlled by regulation of the rate of polyamine acylation and oxidation, resulting in interconversion of spermidine into putrescine. Spermidine/spermine-*N*-acetyltransferase (SSAT), the rate limiting enzyme of the polyamine interconversion pathway, is rapidly induced in the brain by several stimuli [14,19]. The resultant increase in putrescine levels after ischemia might also be due to a temporary activation of SSAT. This pathway may also contribute to ischemia related neurodegeneration as a toxic product of the polyamine oxidation reaction, hydrogen peroxide, may participate in neuronal damage. This may also explain that maintenance of some oxygen supply to the cerebral tissue as in the penumbra region or reestablishment of such supply in ischemia-reperfusion is necessary to induce an increase in putrescine levels after cerebral ischemia.

Focal cerebral ischemia due to MCA occlusion differs from global or forebrain ischemia in two major respects [12,16]. The reduction in CBF caused by MCA occlusion is usually less severe than that observed during global or forebrain ischemia models. A focal ischemic lesion can be considered to consist of a central core of densely ischemic tissue and perifocal areas where the ischemia is less dense [36]. The less ischemic region, penumbra, consist of perifocal tissues containing electrically unexcitable but essentially viable cells that could be made to function by revascularization of the tissue. However, this condition is not static because the reduction of blood flow may progress and involve penumbral and even more peripheral areas [35].

Ischemic brain edema is a major factor in the progression of ischemic injury, which can cause progressive microcirculatory compression and may involve mixed components of cellular (cytotoxic) and vasogenic edema. In the present study, we have shown that the greatest edema was observed in the densely ischemic region with intermediate levels in the penumbra region. However, putrescine levels were not increased in the densely ischemic region, suggesting that the polyamine putrescine may not be involved in initial brain edema formation after permanent focal ischemia. As polyamine metabolism is proposed to be an important pathway in the development of late vasogenic edema and delayed neuronal death after transient ischemia [21,29], increased levels of putrescine may play a role in the development of later vasogenic edema after permanent focal ischemia. It has been shown that increased ornithine decarboxylase activity and polyamines in neurons, as well as polyamine-dependent Ca^{2+} influx play a particular role in mediating the physiological effects of excitotoxicity at the NMDA receptors [25]. Polyamines have also been implicated in blood-brain barrier (BBB) breakdown and vasogenic brain edema induced by cold injury [24], global and focal cerebral ischemia [26,32,33], and traumatic brain injury [1]. Furthermore, irreversible inhibition of ODC activity by difluoromethylornithine (DFMO) improved histopathological outcome after transient global ischemia [21], attenuated BBB breakdown in permanent focal ischemia [33] and edema formation after transient ischemia [32] and traumatic brain injury [2].

In contrast to studies mentioned above and many others, there are several important reports [13,27] suggesting that the polyamine putrescine is not neurotoxic in all situations and induction of the ODC-polyamine pathway may be an adaptive response to harmful stimuli to the nervous system. Gilad and Gilad [13] have shown that systemic polyamine injection reduces neuronal damage after transient global ischemia in gerbils. Moreover, recently it has also been demonstrated that an extensive stimulation of ODC and accumulation of endogenous putrescine does not effect the long-term recovery, nor increase neuronal damage after incomplete global ischemia in transgenic mice overexpressing the human ODC gene [27]. However, as we stated earlier in the discussion, the interconversion pathway of polyamines may also play an important role independent from ODC induction. A recent study reported by Cockroft et al. [6] has shown that aminoguanidine, an inhibitor of polyamine oxidation, reduces infarct size after focal ischemia in rats.

This study demonstrated that extracellular polyamine levels are not altered within 6 h of permanent focal ischemia. These findings are consistent with a study reported by Gilad et al. [15] in which no significant change of extracellular polyamines has been found in the transient forebrain ischemia model. It has been shown that polyamines may be released under in vitro conditions from neurons and/or glial cells into the extracellular compartment [18]. It might be difficult, however, to detect them in the extracellular compartment in in vivo conditions because of their increased cellular uptake. As a transient elevation of ODC activity has been shown to peak at 4–8 h of reperfusion, delayed changes in extracellular polyamine levels may occur over 3 h of reperfusion. Beside the ischemic injury, placement of microdialysis probes may also cause an increased efflux of polyamines as has been shown in recent studies [5,11]. In a most recent study [5], putrescine levels were not found to be increased significantly whereas spermidine levels increased consistently in rats subjected to MCA occlusion. This increase was observed specially around 9–12 h after MCA occlusion. There are several factors that may effect polyamine levels, such as MCA occlusion techniques, using thermocoagulation for occlusion of MCA, and species differences.

In conclusion, the findings of this present study suggest that induction of the polyamine pathway is dependent on blood flow and maintenance of oxygen supply to the brain after cerebral ischemia. Extracellular levels of the polyamine putrescine do not reflect changes in intracellular levels of putrescine in permanent focal ischemia.

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