^{31}P and ^{23}Na Nuclear Magnetic Resonance Study on Forebrain Ischemia in Rats with Shift Reagent Dy(TTHA)

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^{31}P and ^{23}Na nuclear magnetic resonance (NMR) spectroscopy was employed to study the dynamic changes in intracellular high-energy phosphates and sodium during 15 min of forebrain ischemia and recirculation in *in vivo* rat brain. In the presence of the shift reagent Dysprosium triethylenetetramine-N,N,N′,N″,N‴,N⁗-hexaacetic and [Dy(TTHA)], the sodium peak separated into two peaks, unshifted and shifted. During 15 min of ischemia, the unshifted sodium peak decreased and the shifted sodium peak increased. With recirculation, the unshifted and the shifted sodium peaks returned to the preischemia level within 10 min, but the shifted one increased during 30–60 min. Intracellular high-energy phosphates and intracellular pH (pHi) decreased during 15 min of ischemia and returned to the preischemia levels within 20 min of recirculation. We conclude that the decrease in unshifted sodium peak during ischemia is due to the decrease in subarachnoid sodium and the cellular influx of interstitial sodium would be minimum. The increase in shifted sodium peak during ischemia is considered to be due to the dilatation of cerebral blood vessels and the increase in interstitial sodium which was transported from subarachnoid space. (Key words: forebrain ischemia, sodium, high-energy phosphate, nuclear magnetic resonance spectroscopy)

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In recent years, the ^{31}P and ^{23}Na nuclear magnetic resonance (NMR) spectroscopy has been used to noninvasively investigate the nature of organs[1–6]. However, the conventional ^{23}Na NMR method is unable to distinguish between intracellular and extracellular sodium. Shift reagents were introduced to separate the intracellular and extracellular sodium peaks[7,8]. The shift reagents usually penetrate the extracellular space, but do not cross the cell membrane, and cause a shift in the signal position of the extracellular ions selectively. Although a number of paramagnetic shift reagents have become available, the use of Dysprosium triethylenetetramine-N, N, N′, N″, N‴, N⁗-hexaacetic acid [Dy(TTHA)] is usually preferred, since it is more stable and less toxic[3–5]. Naritomi et al.[3] showed in the gerbil brain that gradual intravenous infusion of Dy(TTHA) caused little suppressive effects on cardio-respiratory

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function and no change in the brain high-energy phosphates. Blum et al.[4,5] reported that Dy(TTHA) was useful to study the dynamic changes in intracellular sodium of rat skeletal muscle and liver during ischemia. In these organs, Dy(TTHA) penetrates the extracellular space freely and the sodium peaks separate into intracellular and extracellular peaks. In the brain, however, the presence of the blood brain barrier (BBB) may cause some limitation to the transport of Dy(TTHA) from the intra- to the extravascular space[9]. As far as we know, there are no reports on the change in sodium peak with Dy(TTHA) in the brain during ischemia. In the present study, we measured cerebral high-energy phosphate compounds and sodium with NMR spectroscopy during forebrain ischemia and recirculation with or without Dy(TTHA), and evaluated the usefulness of ^{23}Na NMR spectroscopy in the brain.

Materials and Methods

Male Wistar rats (250–300g) were fasted for 2 h before use[10]. Each rat was anesthetized with sodium pentobarbital (50 $mg \cdot kg^{-1}$, i.p.). and heparinized (1,000 $unit \cdot kg^{-1}$, i.p.). The rats were mechanically ventilated via an endotracheal cannula with a mixture of 60% nitrous oxide and 40% oxygen, and were maintained normocapnic. The body temperature was maintained at $37 \pm 0.5°C$ throughout the study. The right femoral artery and vein were cannulated for blood pressure monitoring and blood withdrawal/transfusion. Bilateral common carotid arteries (CCAs) were separated gently, and home-made occluders were placed around them, allowing for temporary occlusion. For the ^{31}P and ^{23}Na NMR analysis of the brain, the skin and the temporal muscles were removed from the skull and a surface coil was placed on the skull. Dy(TTHA) was prepared for the shift reagent. TTHA was dissolved in water and titrated with NaOH to pH 7.0. $DyCl_3 \cdot 6H_2O$ was added to this solution to a final concentration of 150 mM. The TTHA-to-Dy ratio was maintained at slightly greater than 1.0. After readjusting the pH to 7.0 with NaOH, 40 mM $CaCl_2$ was added to chelate any excess TTHA. This was helpful in preventing cardiac arrhythmias on infusion of the shift reagent[4]. The pH of the solution was adjusted to a final level of 7.35–7.40[3].

After the magnet was shimmed for maximum uniformity at the observation site, conventional ^{31}P and ^{23}Na spectral measurements were carried out on the brain. Thereafter, a total of 7 ml of 150 mM Dy(TTHA) was slowly infused from the right femoral vein for 1.5 h excepting the Dy(TTHA)-untreated rats. After 1.5 h, the ^{23}Na spectra showed two peaks (unshifted and shifted peaks) which reached equilibrium. Then 0.2 $ml \cdot h^{-1}$ of infusion was continued to maintain the Dy(TTHA) concentration at a constant level.

In ^{23}Na NMR analysis, the rats were divided into two groups; Dy(TTHA)-untreated (n=6) and Dy(TTHA)-treated (n=5). In addition, to investigate the relation between intravascular sodium and the intensity of the shifted sodium peak, ischemia was induced after 15 min by an injection of 1 $mg \cdot kg^{-1}$ of papaverine hydrochloride, a potent vasodilator, in the Dy(TTHA)-treated rats (n=4). Forebrain ischemia was induced through occlusion of the CCAs combined with a reduction in mean arterial prressure to approximately 40 mmHg by means of blood withdrawal. Under these conditions EEG activity rapidly became isoelectric. After 15 min of ischemia, occlusion of the CCAs was released and the blood was reinfused. The NMR spectra were monitored every 5 min during ischemia and

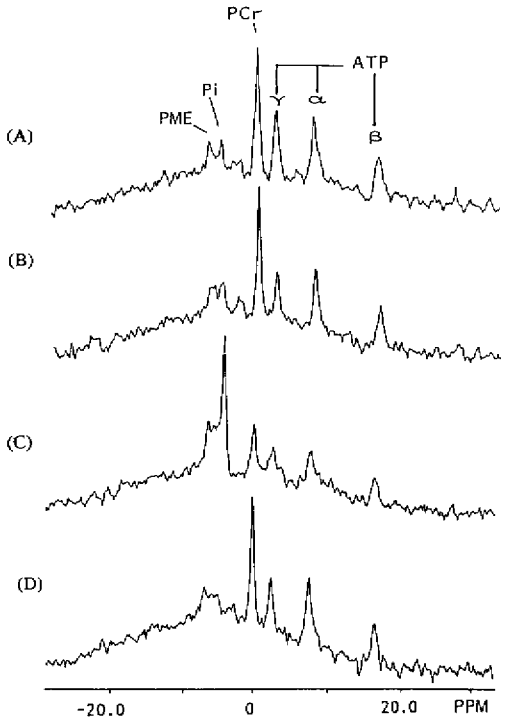

Fig. 1. ^{31}P NMR spectra obtained from *in vivo* rat brain during ischemia and recirculation. (A) control spectrum. (B) 1.5 h after infusion of the shift reagent Dysprosium triethylenetetramine-N,N,N′,N″,N‴,N⁗-hexaacetic acid [Dy(TTHA)]. (C) 15 min after the induction of ischemia. (D) 30 min after recirculation.

60 min of recirculation.

The ^{31}P and ^{23}Na NMR spectra were recorded at 80.65 MHz and 52.96 MHz, respectively, with a 4.7 tesla superconductive magnet (Magnex Scientific, Abingdon, Oxon) which was interfaced with a BEM-170/200 spectrometer (Otsuka Electronics Co., Ltd. Minakuchi, Japan). ^{31}P spectra were acquired with pulse-repetition times of 2.5s; the sum of 120 scans was recorded using 23 μs radio frequency pulses at 5-min intervals. ^{23}Na spectra were acquired with pulse-repetition times of 0.05s; the sum of 2270 scans was recorded using 40 μs radio frequency pulses at 5-min intervals. The peak of phosphocreatine (PCr) and the peak of sodium before Dy(TTHA) infusion were assigned a chemical shift of zero, respectively. The tissue levels of PCr inorganic phosphate (Pi), β-adenosine triphosphate (ATP), and unshifted and shifted sodium peaks were estimated from the area under the peaks. The intracellular pH (pHi) was calculated from the chemical shift (σ) of the Pi peak using the following equation[11];

$$pHi = 6.77 + \log[(\sigma - 3.29)/(5.68 - \sigma)]$$

Chemicals

TTHA was purchased from Dojin Chemical (Kumamoto, Japan). $DyCl_3 \cdot 6H_2O$, papaverine hydrochloride and all other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Statistics

Values were presented as mean ± SEM. Data were examined by analysis of variance using Fishers least significant difference method[12], and $P < 0.05$ was considered as statistically significant.

Results

The ^{31}P NMR spectrum of a typical *in vivo* rat brain is presented in figure 1A. A number of characteristic spectral lines are demonstrated: α, β, and γ phosphorus of ATP, PCr and Pi. After infusion of Dy(TTHA) for over 1.5 h the spectrum is essentially unchanged (fig. 1B). There were no significant differences between the areas under the β-ATP peak before and after infusion of Dy(TTHA). With ischemia, PCr and β-ATP peaks decreased and the Pi peak increased markedly (fig. 1C). With recirculation, PCr, β-ATP and Pi peaks returned to the preischemia levels at 20 min (fig. 1D). Figure 2 shows the percent changes from baseline in β-ATP and the changes of the

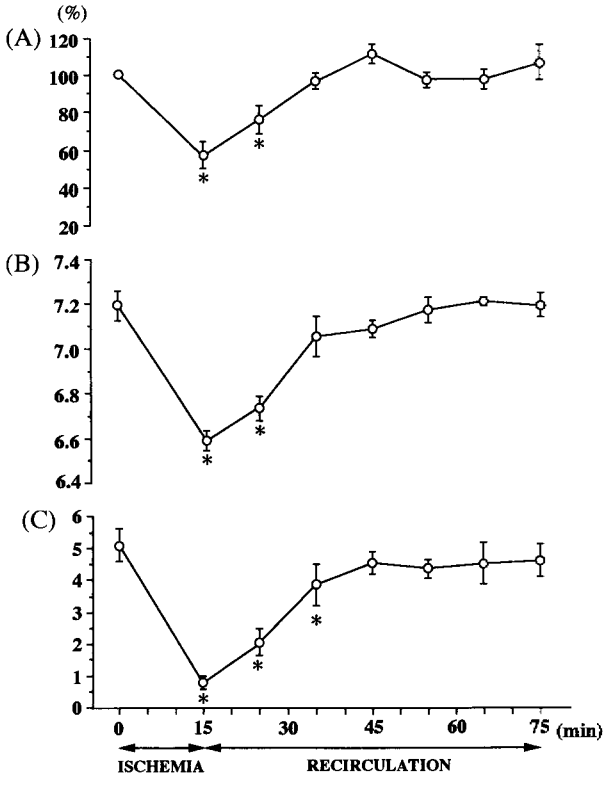

Fig. 2. Changes in β-ATP (A), pHi (B) and PCr/Pi ratio (C) during ischemia and recirculation in rat brain. Values are mean ± SEM. $^*P < 0.05$ compared with preischemia value.

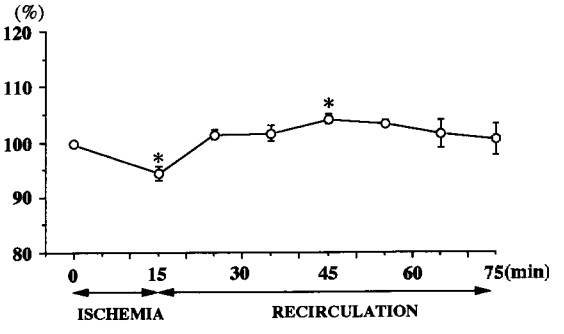

Fig. 3. Changes in sodium peak without shift reagent during ischemia and recirculation in rat brain. Values are mean ± SEM. $^*P < 0.05$ compared with preischemia value.

pHi and PCr/Pi ratio. The β-ATP decreased to 56.4 ± 7.85% at 15 min of ischemia and returned to preischemia values at 20 min of recirculation. The pHi decreased from 7.20 ± 0.07 to 6.59 ± 0.04 at 15 min of ischemia and returned to preischemia values at 20 min of recirculation. The PCr/Pi ratio decreased from 5.10 ± 0.52 to 0.81 ± 0.20 at 15 min of ischemia and returned to the preischemia level at 30 min of recirculation.

Figure 3 shows the percent changes from the baseline of sodium peaks without Dy(TTHA) of *in vivo* rat brain. The sodium peak decreased to 94.5 ± 1.2% at 15 min of ischemia. With recirculation, the sodium peak increased to 104.5 ± 0.8% at 30 min and then decreased. The ^{23}Na NMR spectrum without Dy(TTHA) showed a single spectral line (fig.

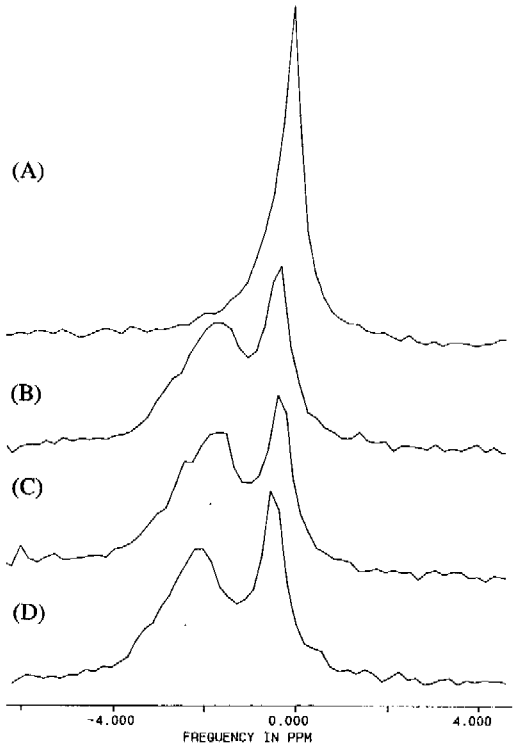

Fig. 4. ^{23}Na NMR spectra with the shift reagent Dysprosium triethylenetetramine-N,N,N′,N″,N‴,N⁗-hexaacetic acid [Dy(TTHA)] obtained from *in vivo* rat brain. (A) before shift reagent. (B) 1.5 h after infusion of shift reagent. (C) 15 min after the induction of ischemia. (D) 30 min after recirculation.

4A). When Dy(TTHA) was infused, the line broadened and separated into two peaks, a broader one which shifted to the left and a narrow one which remained unshifted (fig. 4B). During ischemia, the unshifted peak decreased and the shifted peak increased (fig. 4C). With recirculation, the unshifted peak and the shifted peak returned to the preischemia level and then the shifted peak increased (fig. 4D). Figure 5A shows the percent changes from baseline of the unshifted sodium peaks. The unshifted sodium peak decreased to 91.0 ± 1.5% at 15 min of ischemia. With recirculation, the sodium peak returned to the preischemia value at 10 min and did not change thereafter. Figure 5B shows the percent changes from the baseline of the shifted sodium peak. The shifted sodium peak increased to 106.2 ± 1.4% at 15 min of ischemia. With recirculation, the sodium peak returned to the preischemia value during 10–20 min and increased during the following 30–60 min. The ratio of the unshifted to the shifted sodium peak was calculated after Dy(TTHA) infusion (fig. 5C). The unshifted/shifted peak prior to ischemia was 0.62 ± 0.04. At 15 min of ischemia, the ratio decreased. With recirculation, the ratio returned to the preischemia values.

The percent changes of the shifted sodium peak treated with and without papaverine hydrochloride during forebrain ischemia are shown in fig. 6. In papaverine-treated rats, the shifted sodium peak increased to 103.6 ± 0.7% after 15 min of papaverine injection and did not change thereafter. When ischemia was induced after 15 min of papaverine injection, the shifted peak increased to 107.4 ± 0.6% at 15 min of ischemia. In papaverine-untreated rats, the shifted peak increased to 106.3 ± 1.6% at 15 min of ischemia. No significant differences were present at 15 min of ischemia between papaverine-treated and papaverine-untreated rats.

Discussion

For large organs, a homogeneous distribution of the shift reagent in the extracellular milieu may be difficult to achieve. For example, the equilibration time has been reported to be 2 h in the gerbil brain[3] and approximately 30 min in the frog sartorius[8] and rat calf[4]. In the present study the ^{23}Na peak become separated into two peaks and reached a steady state at approximately1.5 h in rat brain after slow infusion of Dy(TTHA). The infusion of Dy(TTHA) caused no changes in the intensity of the PCr, ATP

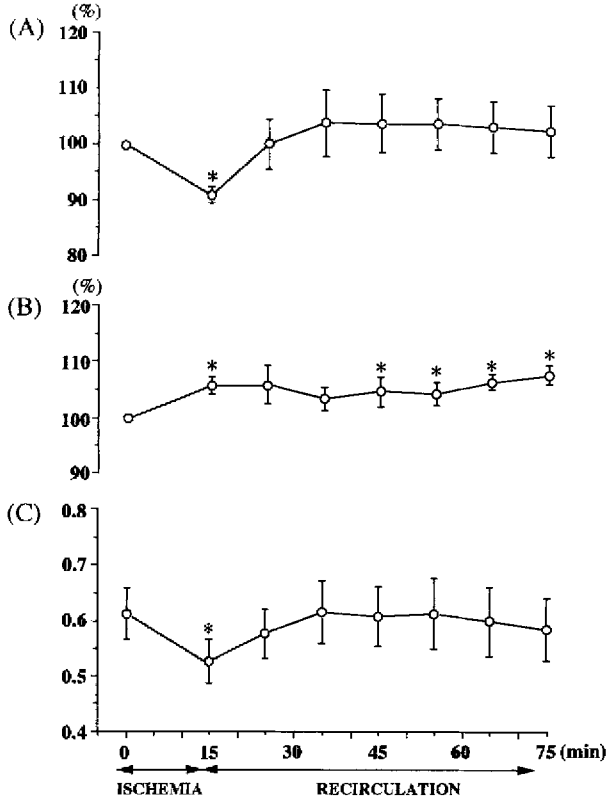

Fig. 5. Changes in sodium peak with the shift reagent Dysprosium triethylenetetramine-N,N,N′,N″,N‴,N⁗-hexaacetic acid [Dy(TTHA)] during ischemia and recirculation in rat brain. (A) the unshifted sodium peak, (B) the shifted sodium peak, (C) the ratio of the unshifted to the shifted sodium peak. Values are mean ± SEM. $^*P < 0.05$ compared with preischemia value.

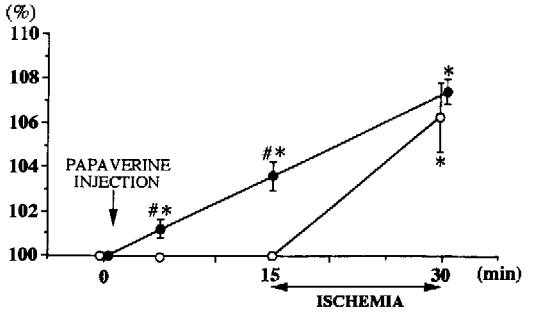

Fig. 6. Changes in the shifted sodium peak treated with or without papaverine hydrochloride during ischemia in rat brain. The shift reagent Dysprosium triethylenetetramine-N,N,N′,N″,N‴,N⁗-hexaacetic acid [Dy(TTHA)] was infused before induction of ischemia in all rats. Values are mean ± SEM. ○; Papaverine-untreated rats. ●; Papaverine-treated rats. $^*P < 0.05$ compared with preischemia value. $\#P < 0.05$ compared with papaverine-untreated rats.

and Pi peaks and pHi, indicating that Dy(TTHA) did not alter the intracellular energy metabolism. The usefulness of Dy(TTHA) for distinguishing the intra- and extracellular sodium in the normal brain was first described by Naritomi et al.[3] and our results were compatible with their report. However, the degree of the sodium shift was smaller than the value reported by Naritomi et al.[3] The possible explanation for this is that the concentration of Dy(TTHA) we used (150 mM) was lower than that in their report (300 mM), and blood was withdrawn when blood pressure was over 120 mmHg. In *in vivo* experiments, the shift reagent may not fully penetrate tissue. Particularly, the distribution of Dy(TTHA) to the extracellular space seems to be restricted in the brain, because of the existing of BBB[9]. In the brain, there are four compartments, the intracellu-

lar, intravascular, interstitial and subarachnoid spaces. It has been reported that Dy(TTHA) is not distributed in the intracellular space[7,8]. After infusion of the Dy(TTHA), plasma sodium concentration was 173.6 ± 3.8 mM. Assuming a baseline intracellular sodium concentration of 30 mM, an intracellular volume of 80%, interstitial sodium concentration of 154 mM, interstitial volume of 10%, cerebral blood volume of 6%, cerebrospinal fluid sodium concentration of 155 mM, cerebrospinal volume under the coil of 4%[13,14], and an average intracellular sodium NMR detectability of 40% in brain *in vivo*[15], we estimated the ratio of [Dy(TTHA) non-diffusible compartments] and [Dy(TTHA) diffusible compartments] in ^{23}Na NMR. The ratio of (subarachnoid + intracellular sodium) to (intravascular + interstitial sodium) was 0.61. In the present study, the ratio of the unshifted peak to the shifted peak was 0.62 ± 0.04. This indicates that Dy(TTHA) penetrates the BBB but is not distributed in the subarachnoid space. Therefore, the unshifted peak would reflect intracellular and subarachnoid sodium, and the shifted peak would reflect intravascular and interstitial sodium.

Eliff et al.[6] reported in the dog brain that the sodium peak without Dy(TTHA) decreased during ischemia. In the present study, the sodium peak without Dy(TTHA) decreased at 15 min of ischemia, which is in agreement with their report. The explanation for this phenomenon was the cellular influx of sodium ion, because in the most complex of tissues the intracellular sodium peak is 40% visible by the NMR methods compared to the extracellular peak which is totally visible[15]. To investigate whether or not the decrease in the sodium peak without Dy(TTHA) during ischemia was due to cellular influx of extracellular sodium, we used the shift reagent. If sodium were transported from the extracellular space to the intracellular space during ischemia, the unshifted sodium peak would increase and the shifted sodium peak would decrease. Blum et al.[4,5] reported that intracellular peak increased and extracellular peak decreased during ischemia in *in vivo* rat liver and skeletal muscle measured by ^{23}Na-NMR. However, contrary to expectations, the unshifted sodium peak decreased and the shifted sodium peak increased during ischemia. Therefore, our result obtained in rat brain was in contrast to the results reported in rat liver and skeletal muscle[4,5].

It has been reported that interstitial sodium is transported to the intracellular space during ischemia[16]. It has also been suggested that subarachnoid sodium is transported to interstitial space and then shifts to the intracellular space during ischemia[17]. As mentioned above, the unshifted peak reflects intracellular and subarachnoid sodium and the shifted peak reflects intravascular and interstitial sodium. In addition, influx of sodium into the intracellular space results in decrease of the visibility of sodium to 40%. Taken together, we supposed that the decrease in subarachnoid sodium contributed chiefly to the decrease in the unshifted sodium peak and the cellular influx of sodium during ischemia would be lower than the values estimated by Hossman and co-workers[16,17]. The increase in interstitial sodium which was transported from subarachnoid space would contribute partially to the increase in the shifted sodium peak. In the present study, forebrain ischemia was induced through occlusion of the CCAs combined with a reduction in mean arterial pressure to approximately 40 mmHg. During ischemia, cerebral blood vessels dilate maximally at a mean arterial pressure of approximately 30 mmHg[18]. Therefore, there is another possibility for the increase in

shifted sodium peak during ischemia, the dilatation of cerebral blood vessels. To confirm this, ischemia was induced in four rats after 15 min of 1 mg·kg ^{1} of papaverine hydrochloride injection. After the injection, the shifted sodium peak (interstitial and intravascular sodium) gradually increased to 103.6 ± 0.7% and reached a plateau at 15 min. When ischemia was induced after 15 min of the papaverine injection, the shifted sodium peak increased to 107.4 ± 0.6% at 15 min of ischemia, which was almost the same value as that of papaverine-untreated rats (106.3 ± 1.6%). These results indicate that the cerebral blood vessels dilate maximally during ischemia.

In early post ischemic brain edema, the degree of ischemia and its duration are important. Mellergard and co-workers[19] reported that 5 min of forebrain ischemia resulted in no observable regional edema during recirculation. When the ischemic period was prolonged to 15 min, edema developed gradually and reached a maximum after 30 min of recirculation. Following 30 min of ischemia, edema was more sustained in time but was not more prominent in magnitude than after a 15-min ischemic insult. Thus, 15 min of forebrain ischemia seems to be adequate for studying early reversible post-ischemic brain edema. Hossmann et al.[17] reported that a decrease in extracellular sodium activities created a concentration gradient between blood and brain during ischemia, and that a passive equilibration along the concentration gradient between the extracellular space and blood occurred during the early recirculation phase. In the present study, the sodium peak without Dy(TTHA) progressively increased and reached the highest value at 30 min of recirculation, which was significantly higher than the preischemia value. In the presence of Dy(TTHA), the unshifted sodium peak returned to preischemia value within 10 min of recirculation but the shifted sodium peak increased during 30–60 min of recirculation. Mellergard et al.[19] reported that following 15 min of forebrain ischemia, brain edema reached a maximum level after approximately 30 min of recirculation and normalized after 180 min of recirculation. Taken together, sodium retention in the interstitial space may contribute largely to the increase in the shifted sodium peak during 30–60 min of recirculation.

In conclusion, the shift reagent Dy(TTHA) could penetrate the BBB but was not distributed in the cerebrospinal fluid. The unshifted peak reflected intracellular and subarachnoid sodium peaks and the shifted peak reflected interstitial and intravascular sodium peaks. The differences in the distribution of Dy(TTHA) in the brain is responsible for the different profile of sodium metabolism during ischemia in other tissues.

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