Metabolism of ^{99m}Tc-Ethylcysteinate Dimer in Infarcted Brain Tissue of Rats

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Brain SPECT with ^{99m}Tc-ethylcysteinate dimer (^{99m}Tc-ECD) reveals a subacute cerebral infarct as a hypoactive area, even in the presence of postischemic hyperperfusion. The brain retention of ^{99m}Tc-ECD depends on hydrophilic conversion mediated by enzymes, and impaired enzymatic trapping is hypothesized to depress the retention efficiency in the infarcted region. The aim of this study was to determine whether the metabolic rate of ^{99m}Tc-ECD is actually reduced in infarcted brain tissue. **Methods:** In 50 mmol/L phosphate buffer (pH 7.4), ^{99m}Tc-ECD was incubated for 30 min with homogenates of rat brain tissue with and without triphenyltetrazolium chloride (TTC) staining. The ratio of polar products was determined by thin-layer chromatography as a function of incubation time, and metabolic rates were obtained. Permanent focal ischemia was induced by occlusion of the right middle cerebral artery (MCA) in rats. The brain was removed 24 h after MCA occlusion, and the infarcted area was defined by TTC staining. The metabolic rate of ^{99m}Tc-ECD was determined in homogenates of infarcted tissue, contralateral noninfarcted tissue, and tissue sampled from sham-operated rats. The infarct volume was measured by direct and indirect methods to assess volume expansion caused by edema, and the metabolic rate in infarcted tissue was corrected for the effect of edema. **Results:** TTC staining had no effect on the metabolic rate of ^{99m}Tc-ECD. The metabolic rates in the infarcted tissue were 0.222%/min ± 0.054%/min and 0.285%/min ± 0.064%/min before and after correction for edema, respectively. These rates were significantly lower than those in the contralateral noninfarcted tissue (0.426%/min ± 0.028%/min) and the tissue sampled from the sham-operated rats (0.439%/min ± 0.031%/min). No substantial difference in rates was observed between the contralateral tissue and the tissue from the sham-operated rats. **Conclusion:** The results of this study showed that infarction decreases the activity of enzymes that mediate the hydrophilic conversion of ^{99m}Tc-ECD in the brain and suggest that reduced metabolic activity is related to decreased accumulation of ^{99m}Tc-ECD in hyperperfused infarcts.

Key Words: ^{99m}Tc-ECD; metabolism; cerebral infarction; animal study

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Technetium-99m-ethylcysteinate dimer (^{99m}Tc-ECD) administered intravenously is taken up by the brain according to regional cerebral blood flow. Radioactive materials are retained for a relatively long period in the brain, although they are removed rapidly from the blood. ^{99m}Tc-ECD provides high-quality SPECT images with low background activity. In addition, it has high in vitro stability, which offers substantial convenience in clinical practice. Because of these characteristics, ^{99m}Tc-ECD is accepted as an excellent brain perfusion agent (*1–5*).

The retention of radioactive materials after the administration of ^{99m}Tc-ECD is thought to be caused by enzymatic trapping (*3,6–8*). ^{99m}Tc-ECD is a small lipophilic molecule that crosses the blood–brain barrier. It is hydrolyzed to ^{99m}Tc-ethylcysteinate monomer (^{99m}Tc-ECM) in the brain, a process that is mediated by enzymes. Arylesterase and carboxylesterase have been suggested to mediate the hydrolysis in the monkey brain (*8*). ^{99m}Tc-ECM is hydrophilic and does not permeate the blood–brain barrier. The enzymatic hydrophilic conversion causes the retention of radioactive materials in the brain for a long enough period to acquire high-quality SPECT images.

The distribution of radioactivity for ^{99m}Tc-ECD brain SPECT reflects that of blood flow in most clinical situations (*9–11*); however, regional differences in enzymatic activity may cause regional differences in the retention efficiency of ^{99m}Tc-ECD. The occipital lobe has shown relatively high accumulation (*12–15*), which may be caused by high enzymatic activity in the area. The discrepancy between blood flow and accumulation may occur during the subacute phase of cerebral infarction (*10,11,16,17*). SPECT with ^{99m}Tc-ECD commonly reveals the infarcted region as a hypoactive area, even in the presence of postischemic hyperperfusion. This finding indicates reduced retention efficiency in infarcted brain tissue, which may be attributable to an impaired enzymatic system. We assessed the metabolic rate of ^{99m}Tc-ECD in infarcted and noninfarcted brain tissues of the rat. This study aimed to determine whether cerebral infarction reduces the activity of enzymes that mediate the hydrophilic conversion of ^{99m}Tc-ECD.

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MATERIALS AND METHODS

Radiopharmaceutical Preparation

We prepared ^{99m}Tc-ECD using a commercial two-vial kit, as described previously (*8*), and then diluted it with 27 mL phosphate buffer (50 mmol/L; pH 7.4). The concentration of the ECD ligand was 25.2 μmol/L in the final solution, and the concentration of radioactivity was about 37 MBq/mL.

Estimation of Lipophilic Content

The radiochemical purity (RCP) of the ^{99m}Tc-ECD solution was determined by thin-layer chromatography (TLC) (*18*). A solution of >95% RCP was used in the experiments.

The residual lipophilic content after the incubation of ^{99m}Tc-ECD with rat brain tissue was assessed by the same TLC procedure. From the tube containing the solution of radioactive material and brain tissue, 0.5 mL of the sample was transferred into an iced tube with 2.5 mL ethanol. After vortex mixing, the tube was centrifuged at 1500*g* for 10 min, and an aliquot of the supernatant was analyzed by TLC. The radioactivity in the precipitated portion was <1% of the radioactivity in the centrifuged tube and was neglected in the analysis. The ratio of the unmetabolized parent (Rp) was determined as the ratio of the radioactivity of $R_f > 0.4$ to the overall radioactivity. The ratio of metabolites (Rm) was obtained from the RCP and the parent ratio as follows: Rm (%) = 100(RCP − Rp)/RCP. TLC was performed in duplicate to measure both the RCP and the residual lipophilic content after incubation, and the mean value was used for analysis.

Effect of TTC Staining

The metabolic rate of ^{99m}Tc-ECD was estimated in the presence of rat brain homogenates with and without triphenyltetrazolium chloride (TTC) staining, and the effect of TTC staining on the hydrophilic conversion of ^{99m}Tc-ECD was evaluated.

Fifteen male Wistar rats (weight range, 263–279 g) were used. They were decapitated, and the brain was removed quickly. After a brief (<1 min) period of cooling on a bed of ice, a coronal brain slice of 2 mm in thickness was made at the level of the optic chiasm and freed from the dura mater and vascular tissue. Brain slices from five rats were stained for 30 min in a solution of 2% TTC in 50 mmol/L phosphate buffer (pH 7.4) at room temperature. After staining, the excess TTC was drained, and the slices were homogenized in phosphate buffer. Brain slices from five other rats were placed in phosphate buffer without TTC for 30 min at room temperature and then homogenized. For the remaining five rats, brain slices were homogenized immediately after brain cutting.

The ^{99m}Tc-ECD solution was transferred into tubes containing the brain homogenate (final concentration, 0.006 g/mL) in phosphate buffer and incubated at 37°C. The total volume of the incubation solution was 10 mL, and the final concentration of the ligand was 2.52 μmol/L. From the tube, 0.5 mL of solution was sampled at 5, 10, 15, 20, 25, and 30 min after the start of incubation, and the ratio of metabolites in the sample was assessed by TLC. The ratio was plotted against incubation time, and linear regression analysis was performed by the least squares method. The ratio of metabolites at time 0 was set at 0. The metabolic rate (%/min) was determined as the slope of the regression line, and the relationship between the metabolic rate and the method of preparation of rat brain homogenates was investigated. Ratios of metabolites > 15% were excluded from the analysis to assess the initial rate of reaction precisely. In addition, the ^{99m}Tc-ECD solution was placed into tubes containing phosphate buffer without the brain homogenate and incubated for 30 min at 37°C. The lipophilic content was assessed after the incubation without the brain homogenate.

Metabolic Rate in Infarct

Permanent focal ischemia was induced in the rat brain, and the metabolic rates of ^{99m}Tc-ECD in the infarcted and the noninfarcted brain tissues were compared. In 10 male Wistar rats (weight range, 246–302 g), permanent focal ischemia was induced by occlusion of the right middle cerebral artery (MCA), as described by Longa et al. (*19*). Rats were anesthetized by inhalation of 1% halothane. The right common carotid artery was exposed, and all branches of the right external carotid artery (ECA) and all extracranial branches of the right internal carotid artery (ICA) were occluded to reduce collateral flow to the territory of the right MCA. A 5-cm length of 4-0 monofilament nylon suture, with its tip rounded by electric heat, was introduced into the right ECA lumen through a puncture and was advanced gently to the right ICA lumen. When resistance was felt, insertion was stopped, and the suture stayed in the position. Resistance indicated that the tip of the suture had passed the MCA origin and reached the proximal segment of the right anterior cerebral artery, resulting in occlusion of the right MCA and interruption of the intracranial collateral flow to the territory of the right MCA. During the operation, body temperature was measured by a rectal probe and maintained at 37°C with a heating pad.

Rats were decapitated 24 h after the onset of MCA occlusion, and the brain was removed quickly. After a brief cooling period, the whole brain was sliced coronally at 2-mm intervals. Brain slices were freed from the dura mater and vascular tissue and soaked for 30 min in a solution of 2% TTC in phosphate buffer at room temperature.

The infarct volume was measured by the direct and indirect methods (*20*,*21*). All brain slices from individual rats were placed on graph paper after TTC staining and imaged with a digital camera. The images obtained were transferred to a personal computer. The infarcted region in the right cerebral hemisphere was defined as a region unstained with TTC and demarcated for each slice by an operator. The infarcted areas of all slices were summed and multiplied by the slice thickness (2 mm) to determine the infarct volume by the direct method. The noninfarcted volume in the right cerebral hemisphere and the total volume in the left cerebral hemisphere were obtained similarly. The infarct volume determined by the indirect method was calculated as the total volume in the left hemisphere minus the noninfarcted volume in the right hemisphere. The infarct volume estimated by the direct method should be larger than that estimated by the indirect method, a difference that results from edema in the infarcted tissue.

Infarcted tissue was sampled from the right cerebral hemisphere and homogenized in phosphate buffer. Noninfarcted tissue was sampled from the homologous region of the contralateral hemisphere and homogenized in phosphate buffer. The ^{99m}Tc-ECD solution was mixed with infarcted or noninfarcted brain homogenate (final concentration, 0.006 g/mL) in phosphate buffer and incubated at 37°C for 30 min. The final concentration of the ligand was 2.52 μmol/L. From the tube, 0.5 mL of solution was sampled every 5 min, and the metabolic rate was determined by the method described for investigating the effect of TTC staining.

A sham operation was performed on 10 other male Wistar rats (weight range, 249–300 g). A rat underwent the sham operation on

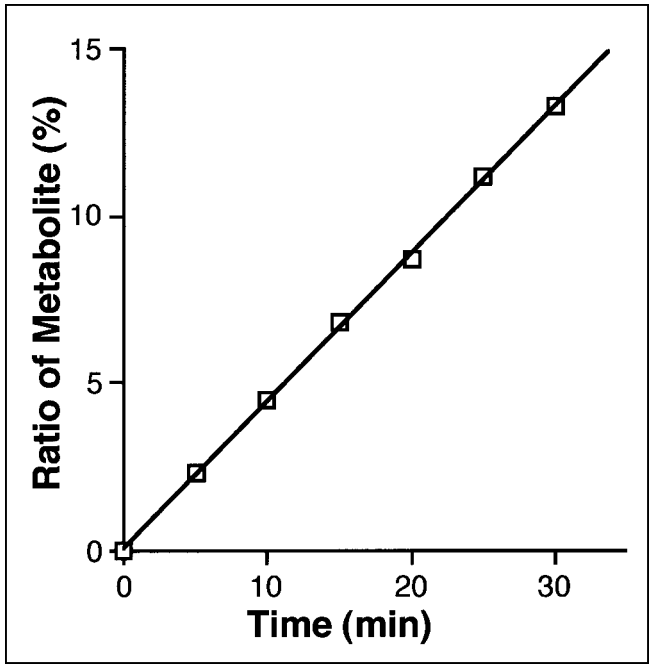

FIGURE 1. Example of temporal profile of ^{99m}Tc-ECD metabolism in TTC-stained brain tissue. Solid line is regression line.

the same day that permanent ischemia was induced in another rat. In the operation, the suture was placed in the right ECA and was not advanced to the right ICA. Otherwise, the procedure was the same as that used for occlusion of the right MCA. Rats were decapitated 24 h after the sham operation, and coronal brain slices were obtained. After TTC staining, brain tissue in the right cerebral hemisphere was sampled from the region corresponding to the infarcted area in the respective rat with permanent ischemia. The tissue was homogenized in phosphate buffer, and the metabolic rate in the tissue from the sham-operated rat was determined.

The metabolic rate in the infarcted tissue was multiplied by the ratio of infarct volume estimated by the direct method to that estimated by the indirect method to correct for the effect of edema. The metabolic rates in the infarcted tissue before and after correction were compared with those in the contralateral noninfarcted tissue and in the tissue sampled from the sham-operated rats.

Statistical Analysis

Values are expressed as mean ± SD. The unpaired Student *t* test was used to determine the effect of TTC staining on the metabolic rates and to compare the metabolic rates for the MCA-occluded rats with those for the sham-operated rats. The paired Student *t* test was performed to compare the metabolic rates in the infarcted tissue with those in the contralateral noninfarcted tissue. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of TTC Staining

Incubation without brain tissue did not change the lipophilic content, indicating the radiochemical stability of ^{99m}Tc-ECD under the experimental conditions. The ratio of metabolites was <15% for every sample in incubation with the brain homogenate, and all points were used for the linear regression. In each experiment, an excellent correlation was found between the ratio of metabolites and the incubation time. In addition, the *y*-intercept of the regression line was close to 0, indicating that the obtained metabolic rate well represented the initial rate of reaction and, consequently, the enzymatic activity (Fig. 1). The resulting metabolic rates are shown in Figure 2. The metabolic rate in the TTC-stained brain tissue was estimated to be 0.441%/min ± 0.038%/min. It did not differ significantly from that in brain tissue placed in phosphate buffer without TTC (0.405%/min ± 0.025%/min) or that in brain tissue homogenized immediately after brain cutting (0.435%/min ± 0.061%/min).

Metabolic Rate in Infarct

Regions unstained with TTC, indicating cerebral infarction, were observed in all rats that underwent occlusion of the right MCA. The infarcted region was confined to the right cerebral hemisphere. The total volume in the left cerebral hemisphere and the noninfarcted volume in the right cerebral hemisphere were 630.4 ± 52.5 mm^3 and 328.1 ± 58.2 mm^3, respectively. Infarct volumes measured by the direct and indirect methods were 387.8 ± 123.7 mm^3 and 302.3 ± 103.4 mm^3, respectively. The ratio of the infarct volume obtained by the direct method to that obtained by the indirect method, a value reflecting volume expansion caused by edema in the infarcted tissue, was 1.298 ± 0.110. There was no evidence of infarction in the sham-operated rats.

Because the ratio of metabolites was <15% in every sample, all points were used in the linear regression in each experiment (Fig. 3). A significant reduction in metabolic rate was observed in the infarcted tissue, even after correction for edema (Fig. 4). The metabolic rates in the infarcted tissue were 0.222%/min ± 0.054%/min and 0.285%/min ± 0.064%/min before and after correction, respectively. These rates were lower than those in the contralateral noninfarcted

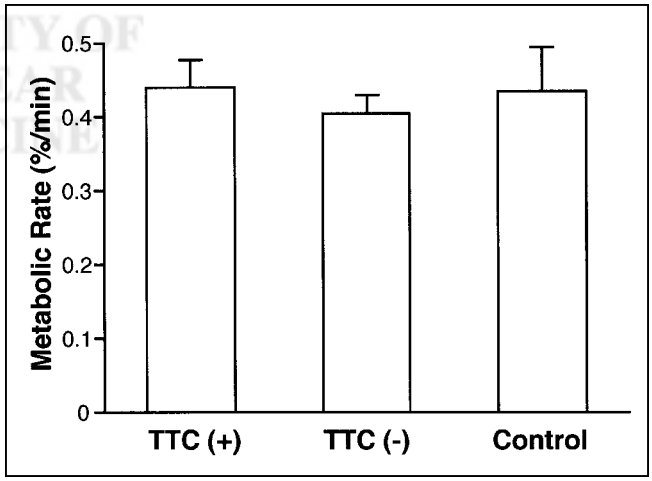

FIGURE 2. Effect of TTC staining on metabolic rate of ^{99m}Tc-ECD. TTC (+) = rat brain tissue stained with TTC; TTC (−) = rat brain tissue placed in phosphate buffer without TTC; Control = rat brain tissue homogenized immediately after brain cutting.

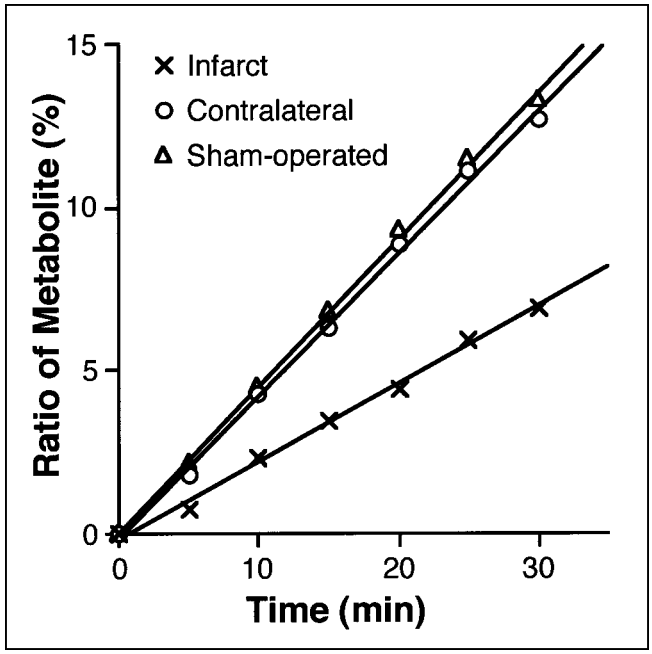

FIGURE 3. Example of temporal profiles of ^{99m}Tc-ECD metabolism in infarcted brain tissue, contralateral brain tissue, and brain tissue from sham-operated rat. Solid lines are regression lines.

tissue (0.426%/min ± 0.028%/min; $P < 0.0001$ before correction; $P < 0.001$ after correction) and those in the tissue sampled from the sham-operated rats (0.439%/min ± 0.031%/min; $P < 0.0001$ before and after correction). No substantial difference was found between rates in the contralateral tissue and rates in the tissue from the sham-operated rats.

DISCUSSION

This study was conducted to assess the activity of ^{99m}Tc-ECD metabolism in cerebral infarction. We used permanent focal ischemia of 24-h duration to obtain infarcted brain tissue. Permanent ischemia was induced by occlusion of the MCA, as described by Longa et al. (*19*). In the technique, the origin of the right MCA is occluded with an intraluminal suture, and collateral blood flow is interrupted intracranially and extracranially. The method is less invasive than those requiring craniectomy and appears to closely model cerebrovascular occlusive disease. In this study, cerebral infarction restricted to the right cerebral hemisphere was shown in all rats that underwent right MCA occlusion.

Infarcted brain regions were defined by TTC staining. TTC is colorless in solution and converted to a deep-red compound with enzymes of functioning mitochondria in noninfarcted tissue. The conversion does not occur in infarcted tissue, and the infarcted region remains unstained. The reliability of TTC staining in discriminating infarcted from noninfarcted brain regions has been established (*22*). If the compound produced in TTC staining has an inhibitory effect on ^{99m}Tc-ECD metabolism, the metabolic activity will be underestimated in noninfarcted tissue stained with TTC. The effect of TTC staining on the metabolic rate of ^{99m}Tc-ECD was examined in this study, and no substantial difference in metabolic rate was observed between rat brain tissues with and without TTC staining. TTC appears to have no effect on the hydrophilic conversion of ^{99m}Tc-ECD, and this observation justifies the use of TTC in comparing the metabolic rates of ^{99m}Tc-ECD in infarcted and noninfarcted brain tissues.

The metabolic rate of ^{99m}Tc-ECD was assessed as an initial rate of reaction in the incubation of ^{99m}Tc-ECD with brain homogenates. Because excessive reaction results in underestimation of the reaction rate, ratios of metabolites $> 15\%$ would have been excluded from the linear regression analysis. However, such high ratios of metabolites were not obtained in this study, and the metabolic rate was calculated using all seven points in every experiment. Linear regression was successful, as shown in Figures 1 and 3, supporting the reliability of estimation of the metabolic rate. Moreover, ^{99m}Tc-ECD was stable in incubation without brain homogenates, and the observed decrease in the lipophilic content is considered to represent ^{99m}Tc-ECD metabolism associated with tissue components. Although the ratios of metabolites were $<15\%$ after a 30-min incubation, the in vivo metabolic rate in the brain should be much higher. The rate of reaction mediated by an enzyme is proportional to the concentration of the enzyme. A low concentration of brain homogenates (0.006 g/mL) was used in this study to avoid excessive reaction and to estimate reliably an initial rate of reaction as an indicator of the activity of ^{99m}Tc-ECD metabolism.

Swelling of the infarcted region occurs at an early stage of ischemic brain injury. The enlargement of infarct volume caused by edema appears to reduce the concentration of cerebral enzymes that metabolize ^{99m}Tc-ECD, leading to a

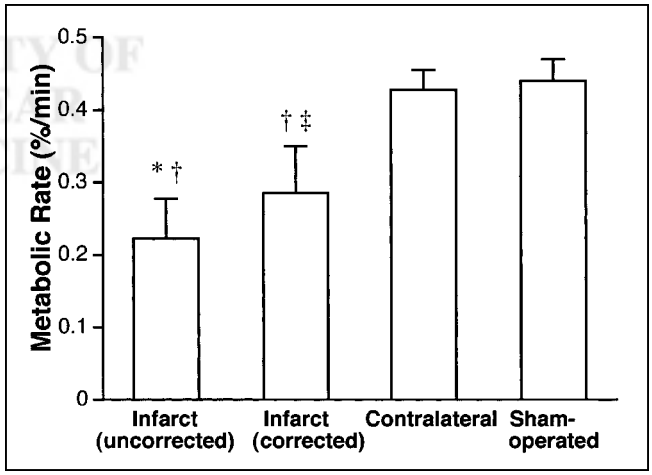

FIGURE 4. Metabolic rates in infarcted brain tissue before and after correction for edema, in contralateral brain tissue, and in brain tissue from sham-operated rats. *$P < 0.0001$ vs. contralateral brain tissue; †$P < 0.0001$ vs. brain tissue from sham-operated rats; ‡$P < 0.001$ vs. contralateral brain tissue.

decrease in metabolic activity, even without the release or inactivation of the enzymes. In this study, we measured the infarct volume by both the direct and the indirect methods to assess the degree of brain edema. The indirect method calculates the infarct volume on the basis of the volume of the surviving normal brain and has been shown to eliminate artificial increases in the infarct volume caused by edema (*21*). The infarct volume measured by the direct method was larger than that measured by the indirect method, indicating that volume expansion occurred in infarcted tissue. The activity of ^{99m}Tc-ECD metabolism is much lower in the blood than in the brain (*8*), and we assumed that the component added to the infarcted tissue in the process of swelling has negligible activity of ^{99m}Tc-ECD metabolism. Because the rate of an enzymatic process is proportional to the concentration of the enzyme, the metabolic rate estimated in infarcted tissue should be inversely proportional to the degree of volume expansion based on the assumption. We determined the ratio of the infarct volume by the direct method to that by the indirect method as an index of volume expansion. We multiplied the metabolic rate estimated in infarcted tissue by this ratio to correct for the effect of edema on the metabolic rate.

The activity of ^{99m}Tc-ECD metabolism was reduced in the infarcted brain tissue in comparison with the contralateral noninfarcted tissue and the tissue of the sham-operated rats. The reduction was still evident after correction for the effect of edema and is not attributable solely to the dilution of enzymes caused by edema. The enzymes that mediate the hydrophilic conversion of ^{99m}Tc-ECD are indicated to be inactivated or released from the brain tissue after cerebral infarction. There may be differences between the human brain and the rat brain in the enzymatic system that metabolizes ^{99m}Tc-ECD (*8*), and the effect of infarction on the metabolic activity may differ also. However, this study suggests that ^{99m}Tc-ECD metabolism may be impaired by ischemic injury, which can lead to a reduced efficiency of ^{99m}Tc-ECD retention. On ^{99m}Tc-ECD brain SPECT performed during the subacute phase of ischemic stroke, decreased accumulation has been shown in infarcted areas with postischemic hyperperfusion (*10,11,16,17*). Reduced metabolic activity appears to be, at least in part, responsible for the discrepancy between blood flow and the accumulation on ^{99m}Tc-ECD SPECT in subacute infarction. We assessed the activity of ^{99m}Tc-ECD metabolism in experiments performed in vitro. Other factors may also be related to decreased retention efficiency in infarcted brain tissue in vivo. It is possible that the reaction rate of residual enzymes alters because of changes in intracellular and extracellular environments. Increased permeability of the blood–brain barrier may accelerate washout of ^{99m}Tc-ECM. Further study is needed to understand fully the cause of decreased accumulation in hyperperfused infarcts.

Brain SPECT with ^{99m}Tc-hexamethylpropyleneamine oxime (^{99m}Tc-HMPAO) often shows a hyperactive area in subacute ischemic stroke (*23,24*). Because increased accumulation is observed in nonviable brain tissue as well as in viable tissue (*25*), the assessment of irreversible brain damage may be disturbed (*26,27*), reducing the accuracy of predicting the clinical outcome (*28,29*). Furthermore, increased accumulation of ^{99m}Tc-HMPAO does not necessarily mean true postischemic hyperperfusion. ^{99m}Tc-HMPAO SPECT tends to overestimate blood flow in subacute infarction because of the elevated efficiency of accumulation in infarcted regions (*30,31*). An in vitro study has also shown that cell membrane disruption increases the accumulation of ^{99m}Tc-HMPAO (*32*).

Infarcted regions show decreased accumulation on ^{99m}Tc-ECD SPECT during acute, subacute, and chronic phases, and this result occurs even without hypoperfusion. The lack of accumulation in an infarcted region would help to assess the full extent of irreversible brain damage and is considered to be an advantage in the clinical use of ^{99m}Tc-ECD (*10,11,33,34*). Early postischemic reperfusion may prevent irreversible damage, and high accumulation on ^{99m}Tc-ECD SPECT has been described in an area where early reperfusion induces hyperperfusion without the development of infarction (*16,35*). ^{99m}Tc-ECD SPECT appears to visualize the cerebral infarct as a hypoactive area and to reflect perfusion in noninfarcted brain regions, including hyperperfused areas. In other words, ^{99m}Tc-ECD is considered to be a perfusion marker of viable brain tissue.

The preservation of enzymatic activity in viable brain tissue appears to be essential for ^{99m}Tc-ECD to be a reliable perfusion marker. Although the metabolic rate was reduced in the infarcted tissue in this study, the metabolic rate in the contralateral noninfarcted tissue did not differ from that in the tissue from the sham-operated rats. The measurement of the metabolic rate in the peri-infarct area may be helpful to confirm the reliability of ^{99m}Tc-ECD in ischemic stroke patients. We examined the metabolism of ^{99m}Tc-ECD in infarcted tissue produced by permanent focal ischemia of 24-h duration. Irreversible neuronal damage occurs through many biochemical events associated with cerebral ischemia, and early reperfusion can modify the course of brain damage. Ischemia of short duration may not affect ^{99m}Tc-ECD metabolism, whereas late reperfusion may induce further damage. On the other hand, longer permanent ischemia than that produced in this study may cause a more severe reduction in metabolic activity. The effect of the duration of ischemia and reperfusion on ^{99m}Tc-ECD metabolism remains to be investigated.

CONCLUSION

We showed that the activity of enzymes that mediate the hydrophilic conversion of ^{99m}Tc-ECD is reduced in infarcted brain tissue of rats, and this finding suggests that the impaired enzymatic activity is related to the decreased accumulation of ^{99m}Tc-ECD in hyperperfused infarcts. Evaluation of the activity of ^{99m}Tc-ECD metabolism in pathologic brain tissue may aid in interpreting ^{99m}Tc-ECD brain

SPECT and assessing its reliability in various clinical situations.

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