Evaluation of Glutathione Localization in Brain Using ^{99m}Tc meso-HMPAO

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The relationship between distribution of ^{99m}Tc meso-hexamethyl propyleneamine oxime (HMPAO) and glutathione (GSH) content was studied in the mouse. **Methods:** The regional distributions of ^{99m}Tc meso-HMPAO and ^{99m}Tc d,l-HMPAO were examined using tissue sampling and autoradiographic methods and were compared with GSH content and distribution by a histochemical procedure. **Results:** The uptake of ^{99m}Tc meso-HMPAO was highest in the cerebellum and lowest in the brain stem, whereas the distribution of ^{99m}Tc d,l-HMPAO was more uniform. The regional distribution of ^{99m}Tc meso-HMPAO in the mouse brain correlated with GSH content (r = 0.787), but that of ^{99m}Tc d,l-HMPAO did not. Treatment with diethyl maleate, a GSH depletor, significantly decreased the ^{99m}Tc meso-HMPAO uptake to 21%–33% of the control in every region, but the reduction of the ^{99m}Tc d,l-HMPAO uptake was moderate (58%–65% of the control). In the autoradiograph, the radioactivity of ^{99m}Tc meso-HMPAO was higher in the gray matter than in the white matter of cerebellum, and more radioactivity was found in cerebellum and in hippocampus than in forebrain without the hippocampus. This pattern of distribution was similar to the histochemical localization of GSH estimated with a sulfhydryl reagent, Mercury orange. **Conclusion:** ^{99m}Tc meso-HMPAO might be used as an imaging agent to assess GSH localization in the brain.

Key Words: regional distribution; ^{99m}Tc meso-hexamethyl propyleneamine oxime; glutathione; brain; histochemical evaluation

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Technetium-99m d,l-hexamethyl propyleneamine oxime (HMPAO) has been used widely as a blood flow imaging agent for the brain. In a previous study (*1*), we found that the uptake of meso-isomer of ^{99m}Tc HMPAO was decreased by treatment of the mouse with diethyl maleate (DEM), a glutathione (GSH) depletor that acts by GSH S-transferase. ^{99m}Tc d,l-HMPAO uptake, however, was not affected.

The exact mechanism of the retention of ^{99m}Tc-HMPAO, either d,l- or meso-, has not been clarified, although the following is proposed. As a lipophilic compound, ^{99m}Tc-HMPAO diffuses across the blood-brain barrier and is converted rapidly to a hydrophilic form that can be retained within the brain tissue. GSH is believed to be responsible for the hydrophilic conversion and retention of ^{99m}Tc HMPAO in the brain (*2,3*). The rate of conversion of ^{99m}Tc d,l-HMPAO to a hydrophilic complex by GSH is much higher than that of ^{99m}Tc meso-HMPAO, the same rate being achieved at only 1/37 of the GSH concentration (*4*). Therefore, the kinetics of ^{99m}Tc d,l-HMPAO are virtually unaffected by the GSH content, and the uptake is determined mainly by the blood flow. On the other hand, the conversion of ^{99m}Tc meso-HMPAO to a retainable form by GSH is slower than the washout of the diffusible form from the brain to blood. Therefore, the uptake of meso-isomer better reflects the GSH content than the blood flow. Based on this hypothesis, we proposed that ^{99m}Tc meso-HMPAO be used as an indicator to assess regional GSH content in the brain, and, accordingly, oxidative stress, which is related to GSH.

In this study, we also examined the regional relationship between ^{99m}Tc meso-HMPAO uptake and GSH content in the brain. Autoradiographic studies were done in nontreated and DEM-treated mouse brain and were compared with distribution of GSH by a histochemical procedure based on the sulfhydryl reagent, Mercury orange (*5–7*).

MATERIALS AND METHODS

Labeling of ^{99m}Tc meso- and d,l-HMPAO

Labeling of ^{99m}Tc meso- and d,l-HMPAO was performed as described previously (*1*). The radiochemical purity checked by three different chromatographic systems (*4*) was >94% for ^{99m}Tc meso-HMPAO and >90% for ^{99m}Tc d,l-HMPAO.

Treatment of Animals and Injection of ^{99m}Tc HMPAO

DEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in corn oil and injected intraperitoneally into five ddY mice, each weighing about 30 g, at a dose of 550 mg/kg body weight. Five control animals were injected with corn oil only. One hour after the DEM treatment, 1.85 MBq ^{99m}Tc meso- or d,l-HMPAO were injected intravenously into each mouse. Thirty minutes after the tracer injection, the animals were killed, and the radioactivity in the brain regions (cerebellum, brain stem, frontal cortex, hypothalamus, striatum, hippocampus, thalamus and cerebral cortex) was measured by an auto-well scintillation counter (Aloka Co. Ltd., Tokyo, Japan).

Determination of Glutathione in Various Brain Regions

DEM was injected intraperitoneally into five ddY mice at a dose of 550 mg/kg body weight. One hour after injection, the animals were killed, the forebrains were removed quickly and the GSH content was measured as described below. Five control animals

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were injected with corn oil only, killed and dissected into cerebellum, brain stem, hypothalamus, striatum, hippocampus, thalamus and cerebral cortex for GSH measurement.

The GSH content was measured as described (*1*), using the high-performance liquid chromatographic electrochemical detection method with a glassy carbon electrode at 1.1 V (cation-exchange column; Shodex SP-825 [Shoko Co. Ltd., Tokyo, Japan]; 8 mm internal diameter × 75 mm; elution with 10 mmol/L citric acid and 10 mmol/L disodium hydrogen phosphate solution adjusted to pH 2.1 with metaphosphoric acid; flow rate 1 mL/min).

Autoradiographic Study of ^{99m}Tc meso-HMPAO-Injected Mouse Brain

One hour after the DEM (550 mg/kg) or corn oil injection, 15 MBq ^{99m}Tc meso-HMPAO were injected intravenously into the mouse. Thirty minutes after the tracer injection, the animal was killed, and the whole brain was removed quickly. The brain was frozen with powdered dry ice, and cut into 30-μm sections with a cryostat (Bright-5030; Shiraimatu Co. Ltd., Osaka, Japan). Coronal and sagittal sections of control brain were contacted, along with those of DEM-treated brain, to an imaging plate (Bas SR-2040; Fuji Film Co. Ltd., Tokyo, Japan) and exposed for 24 h. The imaging plate was processed in a Bio-Imaging Analyzer (Bas-2500; Fuji). The autoradiograms were analyzed using a MacBas (Fuji) to obtain the radioactivity distribution. Sections on glass slides were stained with hematoxyline-eosin for anatomic identification of brain regions.

Histochemical Staining of Glutathione in Mouse Brain Using the Sulfhydryl Reagent Mercury Orange

Staining of GSH was done according to the modified methods of Asghar et al. (*5*), Slivka et al. (*6*) and Pearce et al. (*7*). The animal was killed, and the whole brain removed quickly. The brain was frozen with powdered dry ice, and cut into 8-μm sections with a cryostat (Bright-5030). Coronal and sagittal sections were thawed on glass slides and immediately immersed in 50 μmol/L Mercury orange (1-[4-chloromercuriphenylazo]-2-naphthol; Sigma Chemical Co., Tokyo, Japan) in ice-cold toluene. After 4 min, the slides were rinsed in ice-cold toluene to remove unreacted Mercury orange and then air dried. The slides were viewed with an Olympus microscope prepared for epifluorescence microscopy (BX60; Olympus Optical Co. Ltd., Tokyo, Japan). Fluorescent images were obtained using an Olympus exciter filter (520 nm $< \lambda <$ 550 nm) with emission filter barrier (λ = 580 nm).

RESULTS

Regional Distribution of ^{99m}Tc meso- and d,l-HMPAO in Normal and Diethyl Maleate-Treated Mouse Brain

The uptake of ^{99m}Tc meso-HMPAO showed a heterogeneous regional distribution in the mouse brain. The highest radioactivity was found in the cerebellum and the lowest in the brain stem. The level of radioactivity in the cerebellum was 1.43 times that in the cerebrum. The regional difference of radioactivity was less significant in the ^{99m}Tc d,l-HMPAO-injected mouse brain. In every brain region, the radioactivity of ^{99m}Tc meso-HMPAO was significantly decreased by DEM treatment to 21%–33% of the control, but the decline of ^{99m}Tc d,l-HMPAO uptake was smaller (about 63% of the control) (Table 1).

Regional Relationship Between ^{99m}Tc meso-HMPAO, ^{99m}Tc d,l-HMPAO and Glutathione Content in Mouse Brain

The regional relationship between the uptake of ^{99m}Tc meso-HMPAO and ^{99m}Tc d,l-HMPAO and the GSH content in mouse brain is shown in Figure 1. The regional distribution of ^{99m}Tc meso-HMPAO correlated with GSH content ($r = 0.787$, $P < 0.02$), but that of ^{99m}Tc d,l-HMPAO did not ($r = 0.215$, $P > 0.5$). The highest and lowest GSH contents

TABLE 1
Effect of Diethyl Maleate on Regional Distribution of ^{99m}Tc meso-HMPAO and ^{99m}Tc d,l-HMPAO in the Mouse Brain

		% dose/g tissue	
	Tissue	Control	Diethyl maleate treatment (% of the control)
^{99m}Tc meso-HMPAO	Cerebellum	2.598 ± 0.256	0.552 ± 0.025 (21.2)
	Brain stem	1.607 ± 0.112	0.521 ± 0.047 (32.4)
	Hypothalamus	1.811 ± 0.172	0.516 ± 0.059 (28.5)
	Striatum	1.832 ± 0.107	0.604 ± 0.059 (33.0)
	Hippocampus	2.040 ± 0.144	0.574 ± 0.068 (28.1)
	Thalamus	1.797 ± 0.100	0.520 ± 0.033 (28.9)
	Cerebral cortex	1.726 ± 0.134	0.533 ± 0.036 (30.9)
	Blood	0.665 ± 0.097	0.801 ± 0.051 (120)
^{99m}Tc d,l-HMPAO	Cerebellum	3.272 ± 0.092	2.208 ± 0.094 (65.5)
	Brain stem	3.284 ± 0.212	1.988 ± 0.313 (60.5)
	Hypothalamus	3.384 ± 0.124	2.141 ± 0.058 (63.3)
	Striatum	3.630 ± 0.163	2.367 ± 0.161 (65.2)
	Hippocampus	3.435 ± 0.444	2.168 ± 0.020 (62.8)
	Thalamus	3.651 ± 0.367	2.144 ± 0.099 (58.7)
	Cerebral cortex	3.505 ± 0.503	2.269 ± 0.145 (64.7)
	Blood	1.386 ± 0.066	1.410 ± 0.046 (102)

Data are expressed as mean ± SD for 5 mice per group.

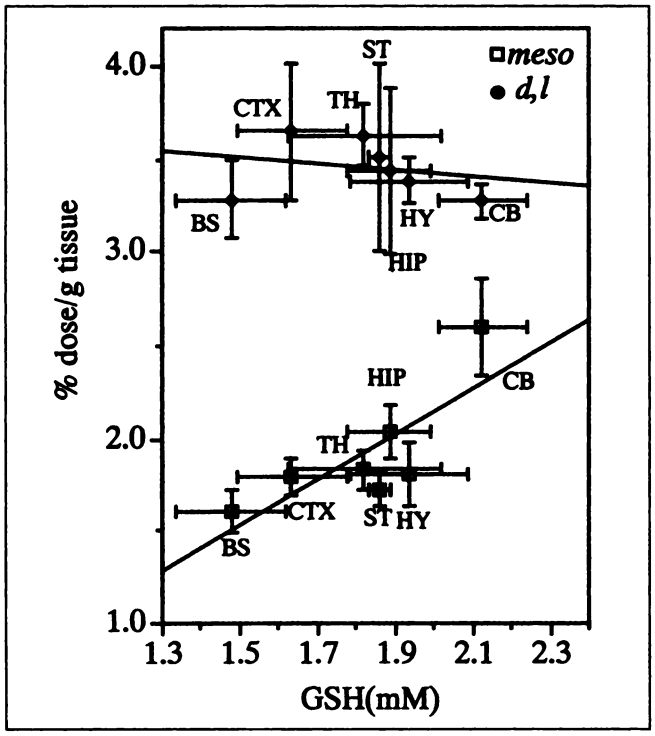

FIGURE 1. Regional relationship between ^{99m}Tc meso- or d,l-HMPAO uptake and GSH content in mouse brain. Radioactivity uptake was measured in five mice by tissue counting 30 min after injection, and GSH content was measured in another five mice. Bars indicate ± 1 SD. Correlation between ^{99m}Tc meso-HMPAO uptake and GSH content was $r = 0.787$, $P < 0.02$, and that with ^{99m}Tc d,l-HMPAO uptake was $r = -0.215$, $P > 0.5$. CB = cerebellum; BS = brain stem; HY = hypothalamus; ST = striatum; HIP = hippocampus; TH = thalamus; CTX = cerebral cortex.

were found in the cerebellum and in the brain stem, respectively, in accordance with the ^{99m}Tc meso-HMPAO uptake. Although the regional distribution of GSH content in DEM-treated mouse brain was not determined, GSH content was reduced to about 30% of the control in the whole brain. The reduction of GSH content was comparable to the finding in a previous report (*1*).

Autoradiographic Imaging of ^{99m}Tc meso-HMPAO Distribution in Normal and Diethyl Maleate-Treated Brain

Coronal and sagittal sections of ^{99m}Tc meso-HMPAO-injected mouse brain are shown in Figure 2. ^{99m}Tc meso-HMPAO radioactivity was distributed throughout the brain. In the sagittal brain section image, more radioactivity was found in cerebellum and hippocampus than cerebrum. The distribution of radioactivity in the cerebellum expressed as photo-stimulated luminescence per millimeter squared was 1.22 times as high as that in the cerebrum. The ^{99m}Tc meso-HMPAO radioactivity was greater in the gray matter than in the white matter of the cerebellum. The ^{99m}Tc meso-HMPAO radioactivity in coronal and sagittal sections was reduced significantly by DEM treatment. Reduced radioactivity was calculated as 33.3% of the control.

Histochemical Localization of GSH in Mouse Brain Using Mercury Orange

Histochemical staining of GSH of the sagittal and coronal sections using Mercury orange is shown in Figure 3. The intensity of fluorescence was greater in the gray matter than in the white matter of cerebral and cerebellum. This tendency was significant in the cerebellum. Higher intensity of fluorescence was found in cerebellum and hippocampus. This distribution pattern of fluorescence was similar to that of ^{99m}Tc meso-HMPAO.

DISCUSSION

In this study, the ^{99m}Tc meso-HMPAO uptake was decreased by the DEM treatment to 21%–33% of the control in all brain regions. The reduction in the uptake of ^{99m}Tc d,l-HMPAO was much smaller. In this experimental situation, GSH content in DEM-treated mouse whole brain was also reduced to about 30% of the control. The uptake of ^{99m}Tc HMPAO is determined by the regional blood flow (reflecting k_1), the rate of back diffusion into the blood (k_2) and the rate of conversion into a hydrophilic form (k_3). Neirinckx et al. (*3*) calculated the rate constants of k_2 and k_3 for ^{99m}Tc meso- and d,l-HMPAO in the human brain. Their findings indicated that the rate-limiting step for the meso-isomer was k_3, whereas that for the d,l-isomer was k_2. This suggests that the k_3 determines the uptake of the meso-isomer. Because GSH is required for the hydrophilic conversion, we have proposed that ^{99m}Tc meso-HMPAO be used as an indicator to assess GSH content and oxidative stress in the brain.

The uptake of ^{99m}Tc meso-HMPAO showed variations in the regional distribution of mouse brain and was well correlated with GSH content, whereas the uptake of ^{99m}Tc d,l-HMPAO was not (Fig. 1). The regional difference of ^{99m}Tc d,l-HMPAO was less significant. This distribution pattern of ^{99m}Tc d,l-HMPAO, which is a blood flow agent, suggests that the ^{99m}Tc meso-HMPAO uptake does not depend on the blood flow.

In terms of the level of ^{99m}Tc meso-HMPAO radioactivity, the regions of the brain examined ranked as: cerebellum > hippocampus, striatum, hypothalamus, thalamus, cerebral cortex > brain stem (Table 1). In GSH content, the regions ranked in the following order: cerebellum > hypothalamus, hippocampus, striatum, thalamus, cerebral cortex > brain stem (Fig. 1). There is a slight discrepancy between investigators regarding the regional GSH content (*8–14*), and our results are also somewhat different. This may be the result of species differences, experimental conditions for preparation of GSH from the samples and difference in expression of GSH unit (mmol/L, μmol/wet weight of tissue or nmol/mg of protein).

In the autoradiographic study, much more radioactivity was found in cerebellum and hippocampus than in the cerebrum, and ^{99m}Tc meso-HMPAO radioactivity was greater in the gray matter than in the white matter of cerebellum (Fig. 2). Excellent gray-to-white contrast in the cerebellum

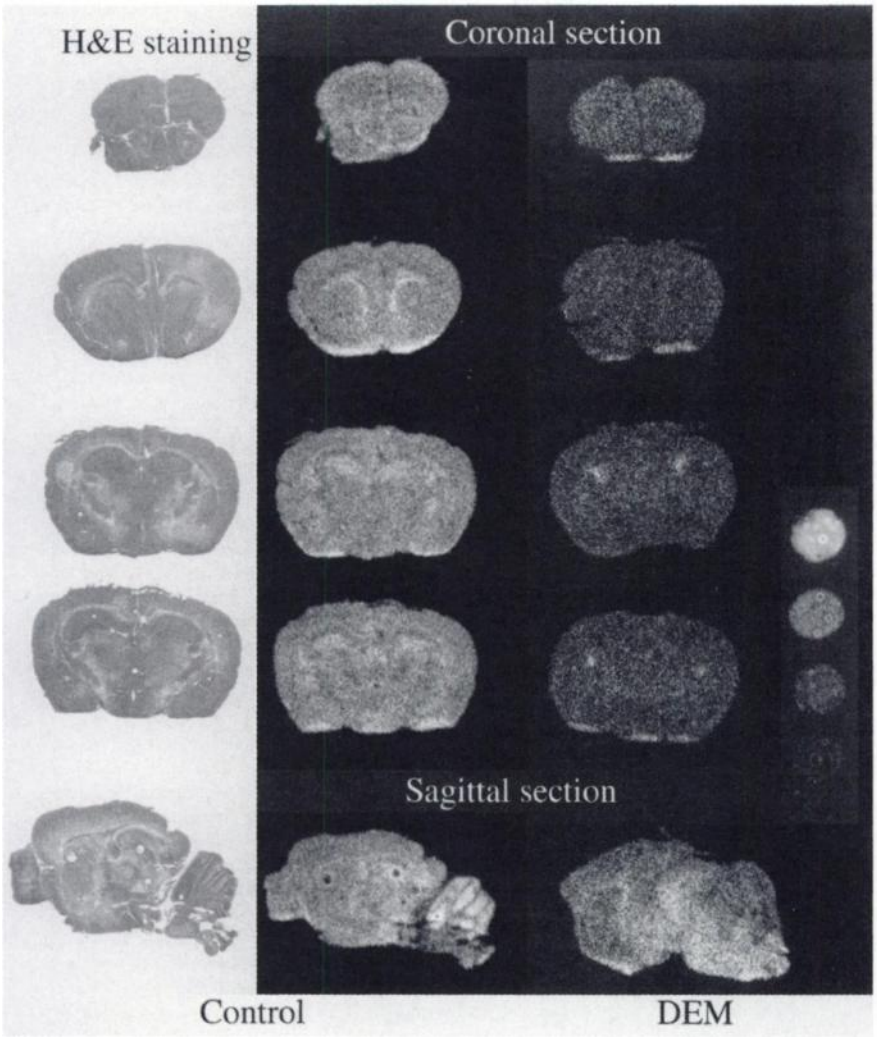

FIGURE 2. Autoradiographic images of ^{99m}Tc meso-HMPAO in control and DEM-treated mouse brain. Control and DEM-treated mouse were injected intravenously with 15 MBq ^{99m}Tc meso-HMPAO. Thirty minutes after tracer injection, animal was killed, and 30-μm coronal and sagittal sections were made by cryostat. Sections were contacted to imaging plate and exposed for 24 h. Equal radioactivity and volume of ^{99m}Tc meso-HMPAO injected into animal was diluted 250–31,250 times with 15% gelatin, and specimen was sliced to 30 μm. Standard sections were also exposed with brain sections for 24 h.

was also reported in other autoradiographic studies (*15,16*). This pattern of radioactivity was similar to the histochemical localization of GSH estimated with Mercury orange (Fig. 3). Mercury orange staining of GSH showed a clear contrast between gray and white matter in the cerebellum. Immunocytochemical staining of GSH localization with an antisera for GSH showed more immunoreactivity in the molecular layers than in the granular layers of the rat cerebellum (*17*).

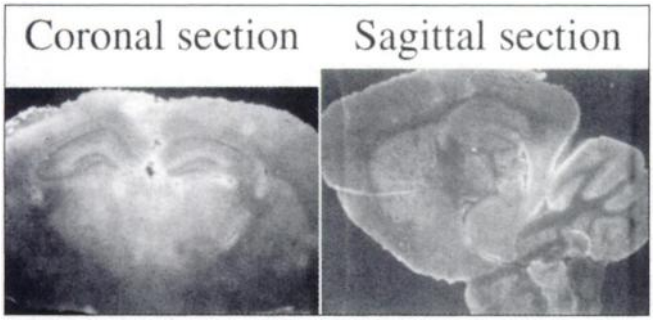

FIGURE 3. Localization of GSH in control mouse brain. Control and sagittal sections stained with Mercury orange and viewed by transmission fluorescence microscopy (×12).

This observation agreed with our result of mouse cerebellum using Mercury orange staining (data not shown).

The histochemical evaluation of GSH was conducted with a sulfhydryl reagent, Mercury orange, in this study. Because this reagent is soluble in toluene, whereas GSH and GSH-Mercury orange complex are insoluble, the diffusion of GSH from the tissue sections during staining was prevented by dissolving the Mercury orange in toluene. This technique is considered GSH-specific, because half of the GSH is stained within 3–4 min whereas several hours are required for reaction with proteins that bear an SH- group. Slivka et al. (*6*) reported that Mercury orange stainable substances were found in monkey cerebellum, hippocampus and cortex. They also demonstrated the reduction of Mercury orange staining in mouse brain after depletion of GSH by pretreatment with DEM. The hydrophilic conversion of ^{99m}Tc meso-HMPAO is also considered specific to GSH, because there is less reactivity of ^{99m}Tc meso-HMPAO to proteins that bear an SH- group.

We found high ^{99m}Tc meso-HMPAO uptake and high GSH content in the hippocampus. Autoradiographic images delineated the shape of the hippocampus (Fig. 2). Differen-

tial vulnerability of CA1, CA2 and CA3 subfields of the hippocampus to hypoxia and ischemia (*18*), reactive oxygen species (*19*) and neuronal poisons (*20*) has been reported. A recent study indicated that heterogeneity of the level of O_2-scavenging enzymes in the hippocampus could contribute to differential sensitivity of CA1, CA2 and CA3 subfields to hypoxia, ischemia, reactive oxygen species and neuronal poisons (*21*). Distribution of ^{99m}Tc meso-HMPAO in the hippocampus may reveal the differential vulnerability among subfields, CA1, CA2 and CA3.

CONCLUSION

The uptake of ^{99m}Tc meso-HMPAO showed variations in regional distribution in mouse brain and was correlated with that of GSH content. The pattern of ^{99m}Tc meso-HMPAO distribution in mouse brain for the autoradiographic study was similar to the GSH localization using a Mercury orange staining technique.

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