Oxido-Reductive State: The Major Determinant for Cellular Retention of Technetium-99m-HMPAO

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Several clinical observations have suggested that HMPAO cerebral uptake might be related not only to regional cerebral perfusion but also to the nature of the lesion. Our aim was to investigate at the cellular level the nature of the process(es) involved in HMPAO accumulation in vitro. **Methods:** Time-course incorporation of HMPAO was studied in a fast-growing human premonocytic line, U937, in a human astrocytic-derived cell line, U373 and a human hybridized endothelial cell line, EaHy926. Minimal differences of HMPAO retention between these cell lines were observed and plateau of $\%U_{HMPAO}$ (cpm cells/cpm standard of injected) were achieved within 2 hr. Because HMPAO cell retention was related to the intracellular content in glutathione, experiments studying effects of redox were conducted by preexposing U937 cells to D,L-buthionine-sulfoximine (BSO), N-acetyl-L-cystein (NAC), D,L dithiothreitol or 2-Mercaptoethanol. **Results:** Overnight incubation with NAC or BSO did not significantly modified the kinetic of ^{99m}Tc-HMPAO incorporation while overnight incubation with NAC resulted in a 2-fold increase in intracellular gluthatione content and overnight incubation with BSO nearly abolished the intracellular glutathione content. At the opposite, presence of these reducing agents in the medium during the experiments completely abolished ^{99m}Tc-HMPAO retention. **Conclusion:** Our data thus provide in vitro evidence to support that overall intracellular retention of HMPAO is more dependent upon the redox activity of the tissue than the intracellular glutathione content. SPECT-HMPAO may accurately reflect regional cerebral blood flow in a normal state but possibly not in all pathological situations in which cell metabolism disturbances are characterized by alterations in the redox status.

Key Words: brain perfusion; technetium-99m-HMPAO; cellular uptake

J Nucl Med 1996; 37:1413–1416

Noninvasive quantitative measurement of regional cerebral blood flow (rCBF) has been a leading aspect of nuclear medicine for decades and is actually used in numerous situations from brain injury to vascular diseases and dementia. Planar dynamic studies using ^{133}Xe initially performed have been replaced by tomographic measurement using PET. The growing interest in clinical setting resulted not only from the development of SPECT with high performances, but even more so from the development of markers that could be used on a daily basis; such as in particular, a radiotracer labeled with ^{99m}Tc the D,L-hexamethylpropyleneamine oxime (HMPAO). This molecule, developed by Neirinckx et al. forms a lipophilic complex with ^{99m}Tc (*1*). HMPAO has been used for many years with the assumption that this molecule passes the blood brain barrier through cell membranes and is retained after intracellular conversion to a hydrophilic form (*2–4*). This intra-cellular conversion of HMPAO has been related to the cellular glutathione content (*5*). Finally, in vivo studies performed in humans have shown that HMPAO brain SPECT imaging was able to adequately reflect rCBF when compared to rCBF measurements obtained from ^{15}O PET imaging (*3,6–8*).

It has been shown that HMPAO uptake in brain tumor may vary from intense focal uptake to photopenic areas (*9–12*) and that high HMPAO uptake may exist in encephalitis (*13*). Investigators also identified the presence of hyper retention of HMPAO in peri-infarct areas early after stroke (*14,15*). Based on our clinical experience in comparing MRI and HMPAO-SPECT and on these published observations, we wondered whether HMPAO tissue retention was only related to local perfusion state, or if additional mechanisms related to the nature of the lesion might be involved. Therefore, our aim was to investigate at the cellular level the nature of the process(es) involved in the accumulation of HMPAO.

MATERIALS AND METHODS

Determination of cellular incorporation of ^{99m}Tc-HMPAO was simultaneously performed to that of ^{111}In-chloride. The cellular retention of ^{111}In-chloride corresponds to the quality control of the experiments based on the fact that the ^{111}In-chloride remains extracellular and therefore, that ^{111}In-chloride cellular content reflects nonspecific cellular uptake and/or alterations of cell membranes. Experiments were also conducted with ^{99m}Tc-O_4 to evaluate the effects of possible free technetium in the medium on measurement of ^{99m}Tc-labeled HMPAO cell content and were compared to ^{111}In-chloride.

Radiopharmaceuticals, Cells and Media

Technetium-99m-HMPAO and ^{111}In-chloride were obtained commercially. The labeling procedure was performed, following the manufacturer's recommendations, in the laboratory immediately before use. In all cases, HMPAO was used within 30 min after preparation and radiochemical purity was verified by TLC according to the manufacturer's recommendations and ranged from 95% to 99%. A fast-growing human premonocytic line (U937), a human astrocytic-derived cell line (U373) and a human hybridized endothelial cell line (EaHy926) were used for these experiments. The U937 and U373 cells were maintained in stationary suspension in RPMI-1640 medium supplemented with fetal calf serum (10%), glutamine (1%) and HEPES (10 m*M*, for U373 cells only). The EaHy926 cells were maintained in DMEM medium supplemented with fetal calf serum (10%), glutamine (1%), HEPES (10 m*M*), Penicillin-Streptomycin (50 U/ml), HAT (1%) In all experiments, fresh stock solutions of HMPAO were prepared with a specific activity of 1 mCi/ml. The solution of pertechnetate was prepared with a specific activity of 1 mCi/ml and ^{111}In-chloride solution with a specific activity of 0.2 mCi/ml.

Radiopharmaceutical Uptake Studies

First, the time-course of incorporation of the markers was studied in the three cell lines used. To study the cellular retention of HMPAO, 1×10^6 cells/ml of medium were placed in conic tubes and exposed ^{111}In-chloride and ^{99m}Tc-HMPAO (50 μl of stock solutions) for specific intervals of times from 0, 15, 30, 45, 60, 90, 120, 240 and 360 min. Control experiments were also

Received Jun. 13, 1995; revision accepted Aug. 18, 1995.

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conducted replacing labeled HMPAO with ^{99m}Tc-O_4. Then, cells were centrifuged at 4°C and washed twice with PBS. Cell pellets were resuspended in 150 μl PBS and then counted in a gamma counter. A total of 50 μl of 1/100 stock solutions of ^{99m}Tc-HMPAO, pertechnetate or ^{111}In-chloride were also counted separately as standard. The cellular uptake of the marker evaluated corresponded to the total counts measured and the results were expressed as the percentage of counts measured in control experiments ($\%U_{^{99m}Tc}$, $\%U_{^{111}In}$, $\%U_{HMPAO}$).

In the experiments aiming at studying the effects of redox status modifications on HMPAO cellular retention, U937 cells were incubated overnight with D,L-buthionine-sulfoximine (BSO, 600 μ*M*), N-acethyl-L-cystein (NAC, 15 m*M*), or 2-Mercaptoethanol (2-ME, 50 μ*M*). Cells were then washed and treated as already described for HMPAO incorporation. In parallel, the effect of reducing agents were analyzed by simultaneous addition of D,L dithiothreitol (DTT, 10 m*M*), NAC (15 m*M*) or 2-ME (15 m*M*) to ^{99m}Tc-HMPAO. BSO and NAC directly modulated the synthesis of gluthation since BSO blocks the gluthation synthetase whereas NAC is a subtrat for glutathion synthesis. In parallel to DTT or 2-ME, NAC can also directly act as a reducing agent able to reduce disulfide bonds. In experiment aiming at modulate glutathion level, we used BSO and NAC whereas in those aiming at modulate the redox potential we used the different reducing agents.

The influence of temperature was analyzed by pre-incubating the cells at 4°C 1 hr before addition of ^{99m}Tc-HMPAO and maintaining this temperature throughout the kinetic study.

In all cases, experiment corresponds to time-course study of cellular retention of marker for 0 to 360 min of exposure repeated 5 to 10 times.

Measurement of Cellular Glutathione Content

After overnight exposure to BSO or NAC, U937 cells were washed with ice-cold PBS, resuspended at 10×10^6 cells/ml of 50 m*M* phosphate buffer pH 7.8 containing 0.1 m*M* EDTA and sonicated. Aliquots of the sonicates were treated with two volumes of ice-cold 3% sulfoxalicylic acid for 15 min on ice. After centrifugation at 10,000 rpm at 4°C the supernatant was immediately frozen for measurement of total glutathione (GSH + GSSG) by the method previously described by Griffith (*16*).

Statistical Analysis

Results are presented as mean ± s.e.m. unless otherwise indicated. Analysis of variance (one-way ANOVA) was used to assess the influence of time on the cellular retention of the different markers tested and to test the significance of differences of retention between the three cell lines tested. To assess the influence of time and the various experimental conditions tested to which cells were exposed (BSO, NAC, T°C), two-way analysis of variance was performed. The Scheffe F-test was used to determine the levels of significance between the parameters tested.

RESULTS

Time-Course Incorporation of HMPAO

The cellular retention of ^{99m}Tc or ^{99m}Tc-HMPAO was measured as a function of time of exposure in parallel to ^{111}In-chloride. In control experiments, $\%U_{^{111}In}$ remained inferior to 0.5% up to 360 min of incubation and was not significantly different as a function of cell type. In control experiments, $\%U_{^{99m}Tc}$ was never significantly different to $\%U_{^{111}In}$.

When cells were exposed to ^{99m}Tc-HMPAO, different time-course profiles were obtained. Minimal differences were observed between time-course profiles of the three cell lines, but the time-course profile was significantly higher with U373 cells than with the EaHy926 cell line (two-way ANOVA, F = 3.282, $p < 0.05$, n = 229) (Fig. 1). After a 120-min exposure to HMPAO, cellular retention of HMPAO in EaHy926 cells reached a plateau at which $\%U_{HMPAO}$ was 18.9% ± 4.1%. For U937 and U373 the maximal uptake of HMPAO is obtained at 240 min at which the $\%U_{HMPAO}$ was 25.4% ± 1.4% for U937 cells and 28.9% ± 2.7%. The incorporation of HMPAO in U937 cells was maintained at 360 min whereas the HMPAO retention in U373 cells slightly declined.

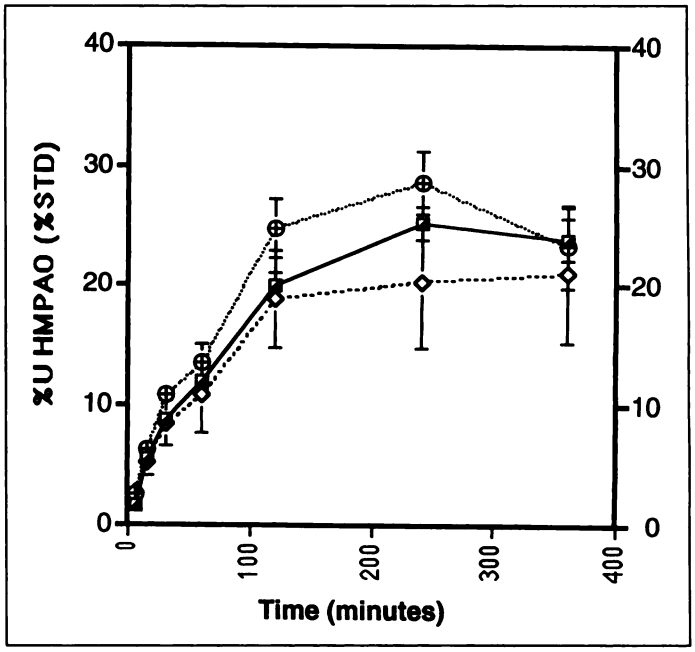

FIGURE 1. Measurements of cellular retention of ^{99m}Tc-HMPAO (%U HMPAO) in EaHy926 (···◇···), U373 (·····⊕·····) and U937 (—◪—) cell lines as a function of time: from 0 to 360 min. Minimal differences of time profiles were noticed between the three cell lines, but the time-course profile was significantly higher with U373 cells than with the EaHy926 cell line (two-way ANOVA, F = 3.282, $p < 0,05$, n = 229).

Effect of Modulation of Intracellular Glutathione Content on HMPAO Cellular Retention

To test the influence of intracellular GSH content on the cellular retention of HMPAO, we modulated the intracellular stock of GSH by overnight pre-exposure of U937 cells to BSO and NAC before incubation with ^{99m}Tc-HMPAO. The intracellular content in total glutathione (0.015 μg/μg of proteins in control cells) was reduced to 0.003 μg/μg proteins after BSO treatment and reached 0.028 μg/μg proteins after exposure to

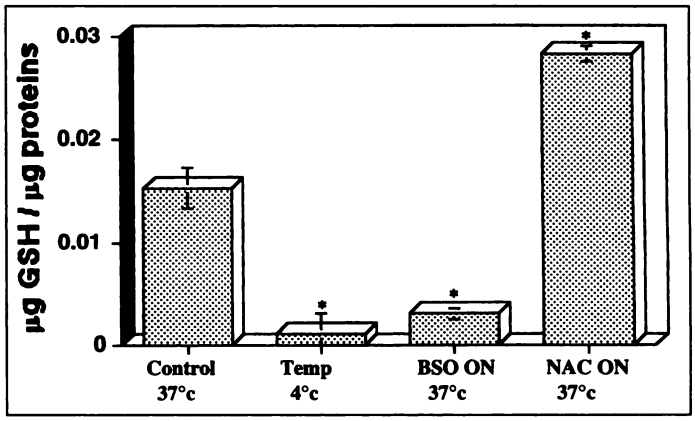

FIGURE 2. Modulation of total intracelluar glutathione content (GSH) in U937 cells under control conditions (37°C), cold (4°C) or after exposure overnight (ON) with DL-buthionine-sulfoximine (BSO: 600μ*M*) and N-acethyl-L-cystein (NAC:15 n*M*).

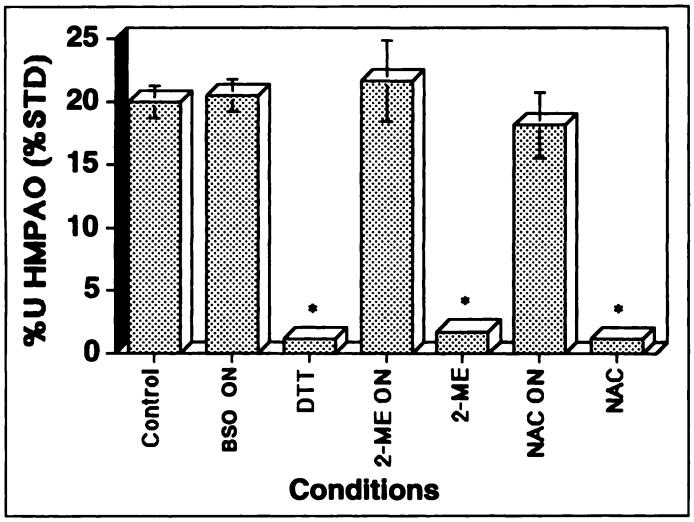

FIGURE 3. Measurements of cellular retention of ^{99m}Tc-HMPAO (%U HMPAO at 120 min) after intra- and extracellular red-ox modifications. Intracellular glutathione modulations were obtained by overnight (ON) pre-exposure of U937 cells to D,L-buthionine-sulfoximine (BSO: 600 μM), 2-mercaptoethanol (2-ME: 50 μM) or N-acethyl-L-cystein (NAC: 15 mM), while extracellular reducing environment was generated by simultaneous addition of D,L-dithiothreitol (DTT: 10 mM), 2-ME (15 mM) and NAC (15 mM) to ^{99m}Tc-HMPAO.

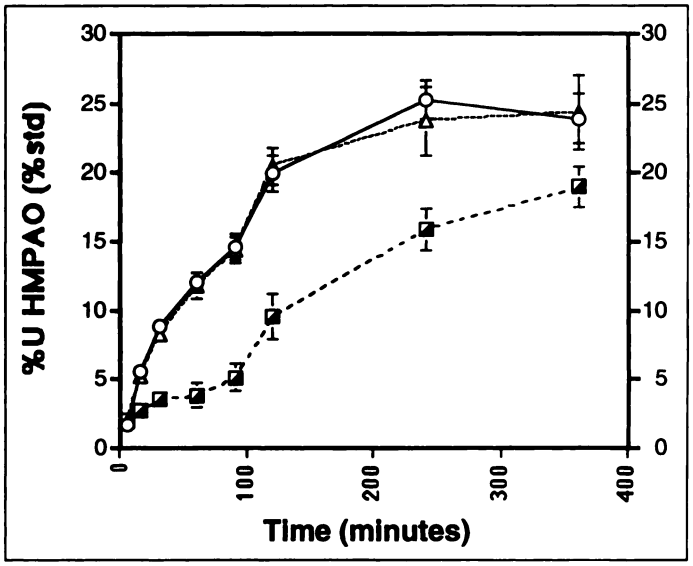

FIGURE 4. Measurements of cellular retention of ^{99m}Tc-HMPAO (%U HMPAO) over a 360-min period during control conditions (—○—) or when U937 cells were to D,L-buthionine-sulfoximine (BSO: 600 μM) (----△----) or 4°C (--◪--).

NAC (Fig. 2). In contrast, pre-exposure of the cells to BSO (depletion of intracellular stock of GSH) or to NAC (increased intracellular production of GSH) did not significantly modify the time-course profile of $\%U_{HMPAO}$ as compared to control conditions. As shown in Figure 3, after a 120-min exposure period to HMPAO, $\%U_{HMPAO}$ was not significantly different in control cells (20.0% ± 1.3%, n = 24) than in cells pre-exposed to BSO (20.5% ± 1.3%, n = 11) or to NAC (18.2% ± 2.6%, n = 6).

Influence of Extracellular Environment on Intracellular HMPAO Retention

As modifications of the intracellular glutathione content did not appear to correlate with the ^{99m}Tc-HMPAO retention, we tested in the U937 cells the influence of modifications in the extracellular redox environment by simultaneous addition of ^{99m}Tc-HMPAO and reducing agents such as DTT, 2-ME or NAC (Fig. 3). While overnight incubation with NAC or 2-ME did not significantly modify the kinetic of ^{99m}Tc-HMPAO incorporation, the presence of these reducing agents in the medium during the experiments completely abolished the retention of ^{99m}Tc-HMPAO. After a 120 min period of exposure to HMPAO, $\%U_{HMPAO}$ incorporated was significantly different from control cells after simultaneous incubation of cells with NAC (1.1% ± 0.1%, n = 8) or 2-ME (1.7% ± 0.2%, n = 5). Similar effects were obtained with DTT (1.1% ± 0.2%, n = 5).

Effect of Temperature on HMPAO Incorporation in U937 Cells

To determine whether in parallel to the HMPAO passive diffusion, there was a selective transport, we analyzed the effects of temperature. Pre-incubation of U937 cells at 4°C 1 hr before addition of ^{99m}Tc-HMPAO and during the whole experiment led to lower $\%U_{HMPAO}$ as compared to control conditions (F-value = 95.7, n = 246, $p < 0.001$) and the curve did not reach a plateau even at long time period such as 360 min (Fig. 4). In contrast, it gradually increased as a function of time and after a 120-min exposure, $\%U_{HMPAO}$ was reduced to 9.6% ± 1.6% thus corresponding to a 52% inhibition compared to control conditions (Fig. 4). After a 360-min exposure, a smaller reduction of ^{99m}Tc-HMPAO cell retention was noticed (20.8%) since a plateau was achieved at 37°C ($\%U_{HMPAO}$ = 24.0% ± 1.8%), whereas the incorporation was still increasing at 4°C ($\%U_{HMPAO}$ = 19.0% ± 1.5%) (Fig. 4).

DISCUSSION

Our data provide in vitro evidence to support that the redox activity is a major determinant of the overall retention of HMPAO. It suggests that in some clinical situations, HMPAO-SPECT may reflect metabolic disturbances related to alterations of the redox status.

The growing interest in functional neuroimaging using SPECT correlates with the improvement in image quality (triple-head camera and fan-beam collimators) and even more so with the introduction of a new class of brain imaging agents labeled with ^{99m}Tc (*17*). The first ^{99m}Tc-labeled marker was the lipophilic compound HMPAO, which has been used for years based on the assumption that this molecule crosses the blood-brain barrier through cell membranes and is retained after intracellular conversion to a hydrophilic form (*2,4,18*). Its intra-cellular conversion has been related to the cellular glutathione content (*5*) and HMPAO brain SPECT imaging has been shown to correlate with rCBF (*7*). But reports have been published showing that HMPAO uptake may be increased in brain tumor (*9–12*) and in presence of encephalitis (*13*). According to the hypothesis that these observations could be a consequence of alterations in cell metabolism and not only in the perfusion state, we have attempted to correlate changes in intra-cellular glutathione content to modifications of the cellular ^{99m}Tc-HMPAO retention in in vitro studies.

Using chemical reagents exerting opposite effect on glutathione content and red-ox state, we demonstrated that cellular retention of HMPAO in U937 cells rather depends from the redox state of the interstitial space than from the intracellular glutathione content. While BSO and NAC have opposite consequences on glutathione levels, the kinetics of ^{99m}Tc-HMPAO retention were not modified accordingly: we observed identical kinetic profile between the different treatments with only minimal inhibition in presence of BSO. In contrast, extracellular addition of reducing agents such as NAC, DTT or 2-ME simultaneously to ^{99m}Tc-HMPAO completely abolished HMPAO incorporation.

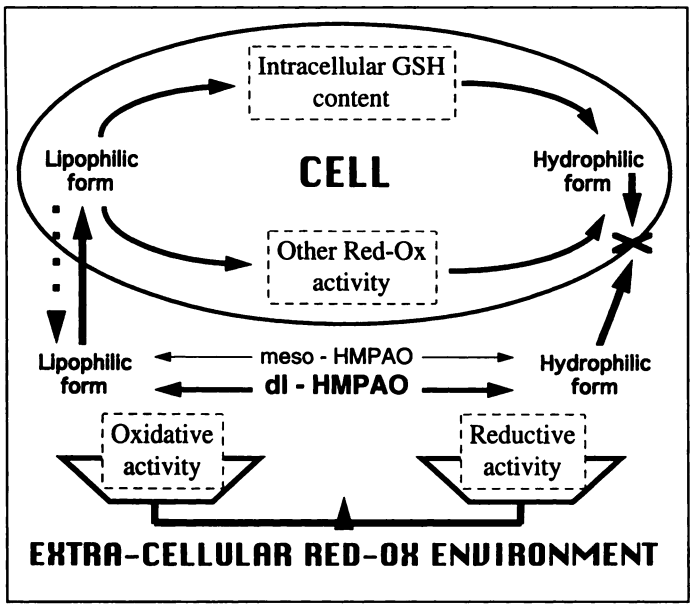

FIGURE 5. Mechanisms involved in HMPAO cell retention.

Neirinckx et al. (*5*) have shown that glutathione could proportionally modulate the initial rate constant of D,L- and meso-HMPAO lipophilic forms conversion into the hydrophilic forms. In their study, the decrease in glutathione (from 160 to 16 μM) induced a decrease in the conversion rate from 0.12 min^{-1} to 0.012 min^{-1}. According to these data we propose that the balance between the two forms of HMPAO is largely influenced by the redox environments (Fig. 5). In contrast to oxidant state which favor the stability of the lipophilic diffusible form, extracellular addition of reducing agent activates the conversion rate of lipophilic form of HMPAO into its hydrophilic form. Consequently, little amount of lipophilic form remains present to diffuse into the intracellular space. Furthermore, after cellular incorporation, only a low amount of glutathione appeared to be sufficient to catalyze the conversion of the lipophilic form into the hydrophilic form which is retained inside the cell.

Thus, total HMPAO incorporation in brain tissue is probably mostly related to total HMPAO available to enter in the cell which depends upon the conversion rate rather than the intracellular GSH content. The decreased cellular incorporation of HMPAO could be primarily the consequence of (1) an increased pericellular reductive activity (i.e., extracellular formation of the hydrophilic complex); and (2) of a modification of the state of equilibrium between D,L- and meso-forms (meso-form being around 10-fold less lipophilic than D,L-form).

In addition to the passive transfer of the lipophilic form of HMPAO, we could not exclude the involvement of an active transport. Experiments performed at 4°C emphasized this hypothesis, since the kinetic profile obtained did not reach a plateau and we observed a strong inhibition in HMPAO incorporation during the first 2 hr. Thus, the kinetics of HMPAO obtained at 4°C could represent the passive transfer and the kinetics obtained at 37°C could represent the sum of both passive and active transport with a saturation at the plateau value. Thus, we are currently investigating the possible presence of transporter(s) for HMPAO. One cannot rule out another hypothesis: alteration of passive transfer due to modification of the membrane fluidity according to a modification of membranar lipid and phospholipids at cold temperature.

CONCLUSION

Our data provide in vitro evidence to support that intracellular retention of HMPAO is overall more dependent on the redox status of the tissue rather than on the intracellular glutathione content. These observations lead us to conclude that SPECT-HMPAO might accurately reflect rCBF in a normal state but possibly not in all pathological situations in which cell metabolism disturbances and alterations of redox state are present. These observations may be of particular importance in some neurodegenerative diseases in which new hypothesis suggest that metabolic disturbance could be the "primum movens".

ACKNOWLEDGMENTS

We thank Professor Pierre Magistretti for helpful comments and stimulating discussions and Nicole Pittet and Laurence Porret for their skillful technical assistance. This research was supported in part by FNSRS grant 31-33781.92 (DOS) and the INSERM (MRJS and BSP).

REFERENCES

1. Neirinckx R, Canning LR, Piper IM, et al. Technetium-99m d,l-HMPAO: a new radiopharmaceutical for SPECT imaging of regional cerebral blood perfusion. *J Nucl Med* 1987;28:191–202.
2. Podreka I, Suess E, Goldenberg G, et al. Initial experience with technetium-99m-HMPAO brain SPECT. *J Nucl Med* 1987;28:1657–1666.
3. Andersen AR, Friberg H, Lassen NA, Kristensen K, Neirinckx RD. Assessment of the arterial input curve for ^{99m}Tc-d,l-HMPAO by rapid octanol extraction. *J Cereb Blood Flow Metab* 1988;8(suppl 1):S23–S30.
4. Lassen NA, Andersen AR, Friberg L, Paulson OB. The retention of ^{99m}Tc-d,l-HMPAO in the human brain after intracarotid blus injection: a kinetic analysis. *J Cereb Blood Flow Metab* 1988;8(suppl 1):S13–S22.
5. Neirinckx R, Burke JF, Harrison RC, Forster AM, Andersen AR, Lassen NA. The retention mechanism of technetium-99m-HMPAO: intracellular reaction with glutathione. *J Cereb Blood Flow Metab* 1988;8:S4–S12.
6. Yonekura Y, Nishizawa S, Mukai T, et al. SPECT with (^{99m}Tc)-d,l-hexamethylpropylene amine oxime (HMPAO) compared with regional cerebral blood flow measured by PET: effect of linearization. *J Cereb Blood Flow Metab* 1988;8:S82–S89.
7. Gemmell HG, Evans NTS, Besson JAO, et al. Regional cerebral blood flow imaging: a quantitative comparison of technetium-99m-HMPAO SPECT with C-15 O2 PET. *J Nucl Med* 1990;31:1595–1600.
8. Murase K, Tanada S, Fujita Hea. Kinetic behavior of technetium-99m-HMPAO in the human brain and quantification of cerebral blood flow using dynamic SPECT. *J Nucl Med* 1992;33:135–143.
9. Lindegaard MW, Skretting A, Hager B, Watne K, Lindegaard K-F. Cerebral and cerebellar uptake of ^{99m}Tc-d,l-hexamethylpropyleneamine oxime (HMPAO) in patients with brain tumor studied by SPECT. *Eur J Nucl Med* 1986;14:39–44.
10. Babich JW, Keeling F, Flower MA, et al. Initial experience with ^{99m}Tc-HMPAO in the study of brain tumors. *Eur J Nucl Med* 1988;14:39–44.
11. Rodrigues M, Fonseca AT, Salgado D, Vieira MR. Technetium-99m-HMPAO brain SPECT in the evaluation of prognosis after surgical resection of astrocytoma. Comparison with other noninvasive imaging techniques (CT, MRI and ^{201}Tl SPECT). *Nucl Med Commun* 1993;14:1050–1060.
12. Schwartz RB, Carvalho PA, Alexander III EA, Loeffler JS, Holman BL. Radiation necrosis versus high-grade recurrent glioma: differentiation by using dual-isotope SPECT with ^{201}Tl and ^{99m}Tc-HMPAO. *AJNR* 1992;12:1187–1192.
13. Schmidbauer M, Podreka I, Wimberger D, Oder W, Koch Gea. SPECT and MR imaging in herpes simplex encephalitis. *J Comput Assist Tomogr* 1991;15:811–815.
14. Moretti JL, Defer G, Cinotti L, Degos J-D, Vigneron N, Ducassou D, Holman BL. "Luxury perfusion" with ^{99m}Tc-HMPAO and ^{123}IMP SPECT imaging during the subacute phase of stroke. *Eur J Nucl Med* 1990;16:17–22.
15. Shimosegawa E, Hatazawa J, Inugami Aea. Cerebral infarction within six hours of onset: prediction of completed infarction with ^{99m}Tc-HMPAO SPECT. *J Nucl Med* 1994;35:1097–1103.
16. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980;106:207–212.
17. Moretti JL, Caglar M, Weinmann P. Cerebral perfusion imaging tracers for SPECT: which one to choose? *J Nucl Med* 1995;36:359–363.
18. Andersen AR. Technetium-99m-D, L-Hexamethylene-propyleneamine oxime (^{99m}Tc-HMPAO: basic kinetic studies of a tracer of cerebral blood flow. *Cerebrovasc Brain Metab Rev* 1989;1:288–318.