脳ミトコンドリア障害のPETによるin vivo評価法とトレーサーの開発

脳ミトコンドリア障害のPETによる in vivo 評価法とトレーサーの開発

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はしがき

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The analysis of brain mitochondrial function using ^{11}C-pyruvate as a tracer for positron emission tomography

Summary

Mounting evidences indicate that the mitochondria play a key role in many diseases of the brain. To analyze mitochondrial function, therefore, provides the important information for diagnosis of the brain diseases. On the other hand, positron emission tomography (PET) is a valuable tool for the study of brain function. However, there is no suitable PET tracer for direct analysis of mitochondrial function. In this study, we employed ^{11}C-pyruvate as a PET tracer, and attempted to analyze mitochondrial function in the brain.

A mitochondrial uncoupler, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 10 μM) induced, within 20 min, the impairment of mitochondrial membrane potential in the cultured hippocampal neurons. FCCP significantly decreased ^{11}C-pyruvate radioactivity indicating that ^{11}C-pyruvate may be useful for analyzing mitochondrial function *in vitro*. We also studied the feasibility of ^{11}C-pyruvate using *in vivo* rat model of excitotoxicity mediated by intrastriatal injection of 3-nitropropionic acid (3-NP), a succinate dehydrogenase inhibitor. The *in vivo* study suggests that: 1) ^{11}C-pyruvate can be useful for detecting the mitochondrial dysfunction in the animal model; and 2) ^{11}C-pyruvate can be used as a PET tracer as follows: a) ^{11}C-pyruvate intravenously injected; and b) 10–20 min after injection, PET scan performed and PET mages analyzed. Finally we examined the possibility of ^{11}C-pyruvate using PET scanner following reversible middle cerebral artery occlusion in monkey. ^{11}C-pyruvate showed the similar pattern as indicated by ^{18}F-fluorodeoxyglucose (^{18}F-FDG). Based on these results, we conclude that, as a PET tracer, ^{11}C-pyruvate is valuable to detect the brain mitochondrial dysfunction *in vitro*

and *in vivo*.

Key words:

mitochondria, PET, ^{11}C-pyruvate, excitotoxicity, ischemia-reperfusion, rat, monkey

Introduction

Excitotoxicity related mitochondrial dysfunction has been implicated in the pathogenesis of several neurological disorders including neurodegenerative diseases, stroke, trauma, and seizures (Choi, 1988). Following mitochondrial dysfunction, the mitochondrial permeability transition pore may open, and cytochrome c will be released to the cytoplasm. These events can participate in the process of delayed neuronal death or apoptosis (De Giorgi et al., 2002) To analyze mitochondrial function, therefore, provides the important information for diagnosis of the brain diseases. On the other hand, positron emission tomography (PET) is a valuable tool for the study of brain function, including cerebral blood flow, oxygen metabolism, glucose utilization, and receptor function (Yamamoto et al., 1999; Yamamoto et al., 2000). However, there is no suitable PET tracer for direct analysis of mitochondrial function. In this study, using hippocampal neuronal culture, rats, and monkeys, we employed ^{11}C-pyruvate as a PET tracer, and attempted to analyze mitochondrial function in the brain.

Methods

Primary culture of hippocampal neuron

Hippocampal neurons were prepared from one-day-old Wistar rats. After removal of the brain under brief anesthesia, the hippocampus was dissected out, and dissociated. Cells were placed on collagen-coated culture dishes (Iwaki 35 mm/Glass Base Dish, Asahi Techno Glass Co., Tokyo, Japan). The culture medium was a mixture of DMEM (50%; Gibco No. 31600, Invitrogen Japan K.K., Tokyo, Japan), HBSS (25%; Gibco No. 11201), and horse serum (25%). Glucose and HEPES were added at a final concentration of 36.1 mM and 23.7 mM, respectively. The pH of the medium was adjusted to 7.2 by adding NaOH. Penicillin-G, streptomycin, and cytosine arabinoside were added to the culture medium. The dish was incubated for a week at 37℃ with a 5% CO_2-containing atmosphere, and was then subjected to the experiment.

Imaging of mitochondrial membrane potential

To measure the mitochondrial membrane potential, in cultured neurons, we used fluorescent dye, rhodamine 123, which accumulates according the transmembrane potential of mitochondria. The dye was loaded into the hippoampal neurons by incubation in artificial cerebrospinal fluid (aCSF) (140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, with a pH 7.2.) containing 1 μM rhodamine 123 (Dojindo, Kumamoto, Japan) in 5%-CO_2 containing atmosphere at 37C for 20 min. The fluorescent images of the rhodamine 123 were observed under a confocal laser microscope (IX 70, Olympus, Tokyo, Japan) equipped with a microlens-attached Nipkow-disk scanner (CSU-10, Yokokawa Electric Co., Tokyo, Japan). The confocal fluorescence images were recorded at video rate with a CCD camera (C2400, Hamamatsu Photonics K.K., Hamamatsu, Japan) combined with an image intensifier (C2400-21SV, Hamamatsu Photonics K.K.), and analyzed with a software, MetaMorph (Universal Imaging Co., Downingtown, PA, USA).

Mitochondrial dysfunction in culture model

To induce mitochondrial dysfunction, in hippocampal culture, we employed a potent

mitochondrial uncoupler, carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP, Sigma Chemical). Cells were incubated in aCSF containing FCCP.

Intrastriatal injection of mitochondrial toxin in rats

Wistar rats (male, 300 g) were anesthetized with i.p. injection of chloral hydrate (400 mg/kg), and mounted on a stereotactic frame (Narishige, Tokyo, Japan). A small burr hole was drilled in the calvarium over the head of the striatum at sites 2.7 mm lateral to and 0.7 mm rostral to bregma. 3-nitropropionic acid (3-NP, 1 M, 3 μl), a succinate dehydrogenase inhibitor, was microinjected through a needle into the striatum over 6 min, and the needle was then left in place for 12 min. After injection, wounds were closed, and animals were returned to their cages. Seven days later, rats were used for the experiment.

Transient focal ischemia in monkey

Male cynomolgus monkeys (*Macaca fasciculoris*), weighing 5.67-6.08 kg, were anesthetized with isoflurane (0.6-0.8%) in a N_2O-O_2 gas mixture (N_2O:O_2 = 7:3), paralyzed with pancuronium bromide (0.05 mg/kg i.m.), and mechanically ventilated. The right middle cerebral artery (MCA) was occluded using a transorbital approach (Hodgins and Garcia, 1970; Takamatsu et al., 2000). The right globe was removed, a craniotomy performed superolateral to the optic nerve, a dura opened, and MCA occluded with a microvascular clip. Three hours after MCA occlusion, the microvascular clip was removed.

Measurement of radioactivity using an imaging plate

Following tracer application, to measure the radioactivity of cultured cells on a glass-base dish and of brain slices, an imaging plate (BAS Phosphor Imaging Plate, Fujifilm, Tokyo, Japan) was used. Glass-based dishes containing cultured cells were directly mounted on an imaging plate for 10 min. In rats with striatal lesion, they were deeply anesthetized, decapitated, brain removed, sectioned into 2 mm-thick coronal slices, and mounted on an imaging plate for 10 min.

Imaging using PET scanner

Serial PET scanning was performed on each monkey with an SHR7700 system (Hamamatsu Photonics, Hamamatsu, Japan). The first scan was obtained before MCA occlusion. The second was obtained 2h after the start of occlusion. The third was obtained after reperfusion. Cerebral blood flow (CBF), cerebral metabolic rate of O_2 ($CMRO_2$), oxygen extraction fraction (OEF), and local cerebral blood volume were measured using the steady-state ^{15}O inhalation method with successive inhalation of trace amounts of $C^{15}O$ (0.8 GBq/min), $^{15}O^2$ (2 GBq/min), and $C^{15}O$ (4 GBq/min). Each gas was administered through a ventilator (f = 15/min, TV = 80 ml).

For analyzing mitochondrial function, one week and one month after the episode of transient MCA occlusion, ^{18}F-fluorodeoxyglucose (^{18}F-FDG, 400-500 MBq) and ^{11}C-pyruvate (1.5-2 MBq) were intravenously injected respectively, and PET scanning was performed.

Histopathological analysis of the brain

One month after reversible ischemia the monkeys were deeply anesthetized, the brain was fixed through transcardial perfusion with a 10% formalin neutral buffer solution, pH 7.4, after saline perfusion at 100 mmHg. The brain was then removed, and 12 coronal sections (2 mm-thick) were made using a brain matrix. Each section was embedded in paraffin wax, and 10 m-thick slices were made and stained with hematoxylin-eosin.

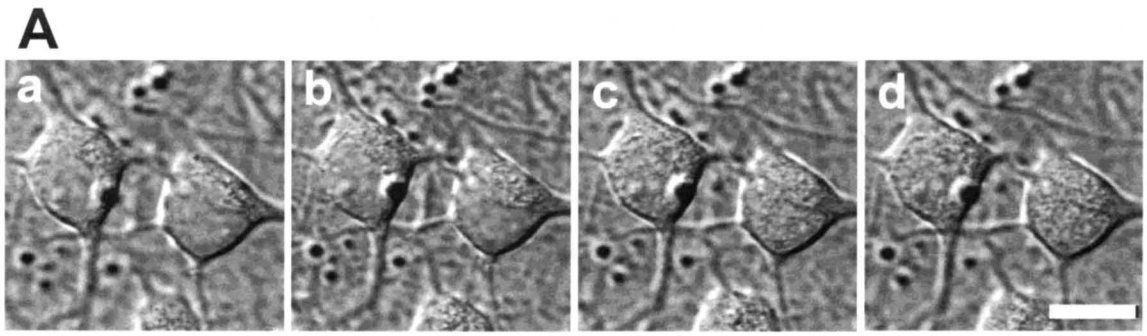
Results

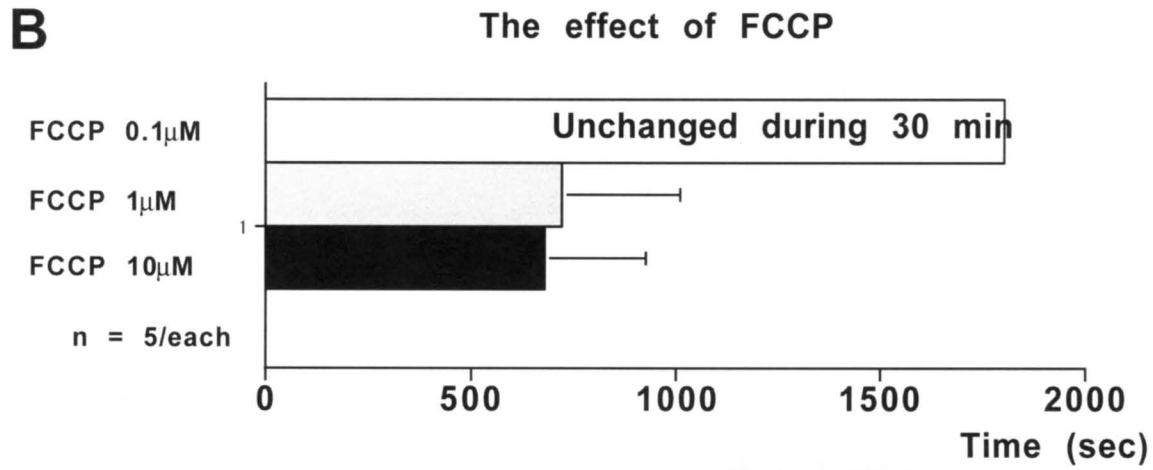
1. FCCP, dose-dependently induces rapid nuclear changes.

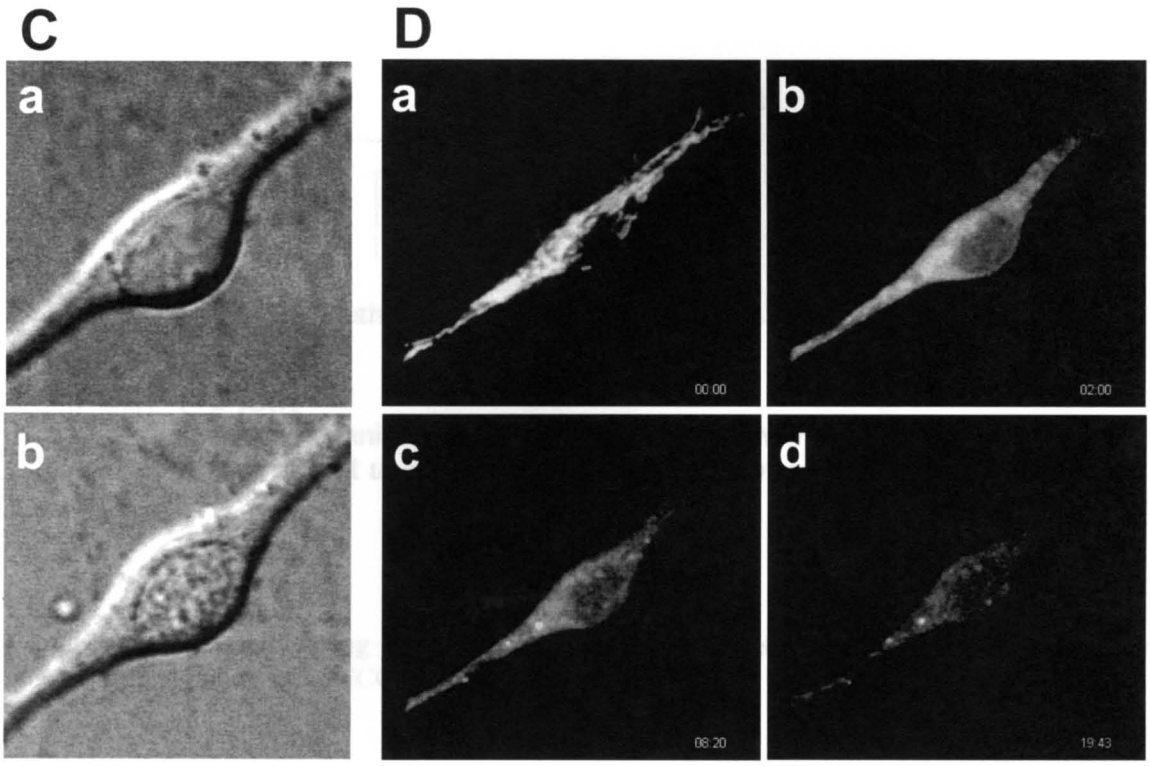
FCCP (10 μM) induced rapid nuclear changes of the neuron (**Fig. 1-A**). The morphological changes were comparable to those induced by glutamate (Ikeda et al, 1996; Yamamoto et al., 1996), which corresponds to nuclear DNA fragmentation (Yamamoto et al. 2003). The nuclear granulation was induced in dose-dependent manner (**Fig. 1-B**). FCCP (10 μM) decreased, within 20 min, fluorescence intensity of rhodamine 123, indicating the impairment of mitochondrial membrane potential in the cultured hippocampal neurons (**Fig. 1-D**), during the same period in which we could observe nuclear changes (**Fig. 1-C**). In the experiment for detecting mitochondrial dysfunction in cultured neurons, we used the above model, i.e. FCCP-induced mitochondrial dysfunction in cultured neurons.

Fig. 1

Sequential changes of rat hippocampal neurons following FCCP exposure (A). Neurons were observed under the video enhanced contrast-differential interference contrast (VEC-DIC) microscope before (a), and at 2.5 min (b), 6 min (c), and 20 min (d) after an application of FCCP (10 μM). Before an application of FCCP, the neurons had large nuclei containing a smooth and amorphous nucleoplasm and one or two nucleoli. Note that FCCP rapidly induced granulation inside the nucleus. Scale Bar = 10 μm. **The effect of FCCP on hippocampal neurons (B).** The graph shows the time at which the granular change was visible (see **Fig.1-Ac**) under the VEC-DIC microscope. **Morphological change in the neuron (C) and sequential changes of in fluorescence intensity of rhodamine 123 (D) in rat hippocampal neurons following FCCP exposure.** Neuron was observed under the VEC-DIC microscope before (**Ca**), and at 20 min (**Cb**) after an application of FCCP (10 μM). In the same neuron, fluorescence intensity was measured under a confocal laser microscope, and recorded before (**Da**), at 2 min (**Db**), 12 min (**Dc**), and 20 min (**Dd**). FCCP (10 μM) decreased, within 20 min, fluorescence intensity of rhodamine 123, during the same period in which nuclear change was observed.


A
a
b
c
d


B
The effect of FCCP
FCCP 0.1μM
Unchanged during 30 min
FCCP 1μM
FCCP 10μM
n = 5/each
0
500
1000
1500
2000
Time (sec)


C
a
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2. ^{11}C-pyruvate detected mitochondrial dysfunction induced by FCCP in cultured neurons.

In cultured hippocampal neurons, mitochondrial dysfunction was induced by an application of FCCP (10 μM). Then we applied ^{11}C-pyruvate (55 MBq) and ^{18}F-fluorodeoxyglucose (10 MBq) to the neurons respectively, and measured the radioactivities of the tracers in the culture dishes using the imaging plate (**Fig. 2, 3**). FCCP significantly decreased ^{11}C-pyruvate radioactivity, and increased ^{18}F-fluorodeoxyglucose radioactivity (**Fig. 4**). The results indicate that the mitochondrial dysfunction induced by FCCP decreased the turnover rate of pyruvate, and enhanced the anaerobic glycolysis of the neurons. ^{11}C-pyruvate can be useful for analyzing mitochondrial function *in vitro*.

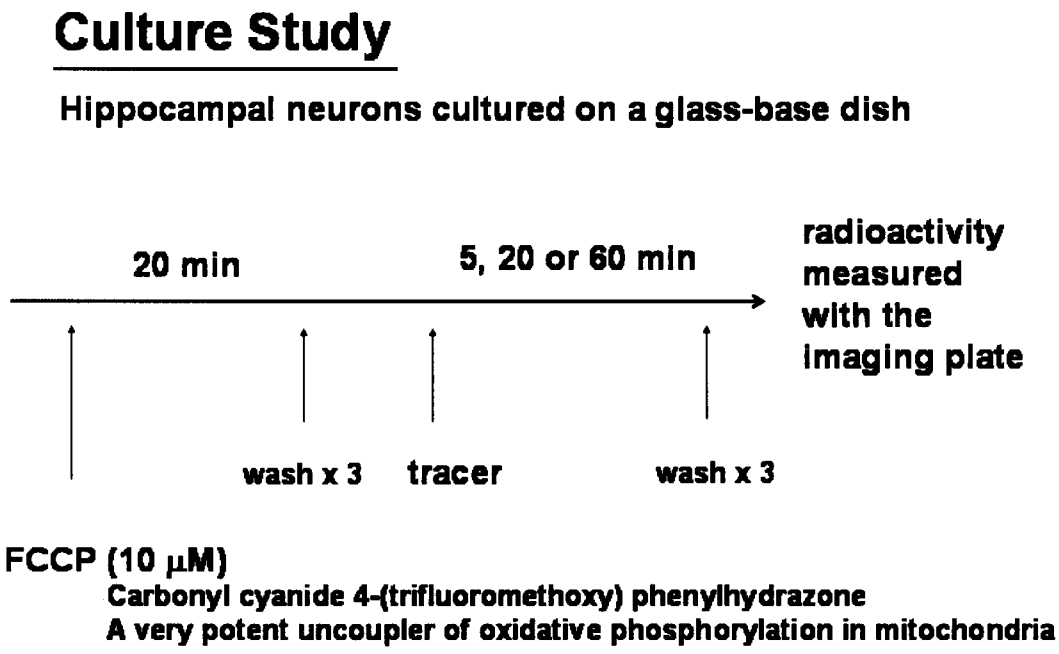

Fig. 2
Protocol for the study using cultured hippocampal neurons. Mitochondrial dysfunction was induced by an application of FCCP (10 μM) (see **Fig. 1**).

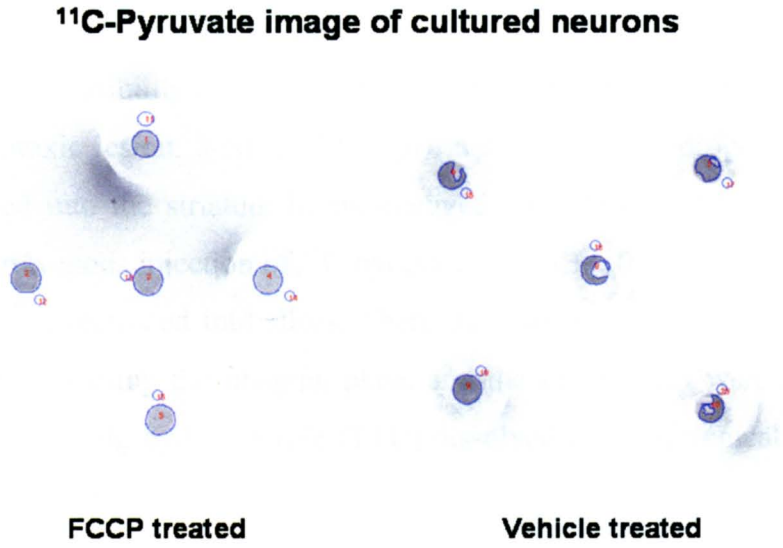

Fig. 3
Representative images of glass-base dish containing cultured neurons with application of ^{11}C-Pyruvate. Note that the circled areas containing cells were darker, indicating higher radioactivity.

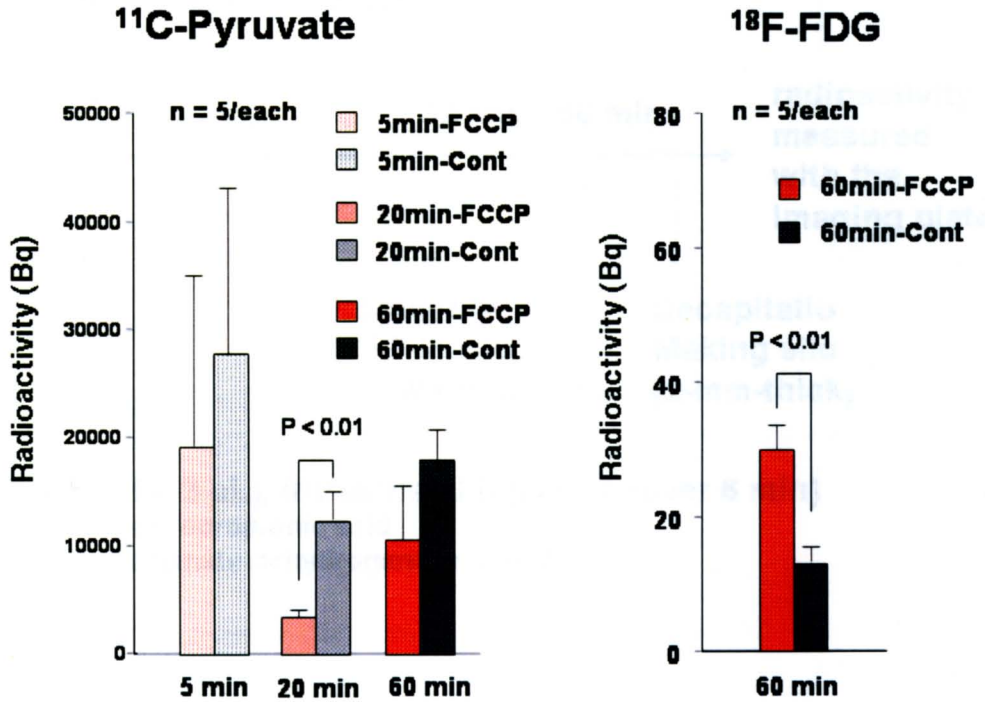

Fig. 4
Radioactivity measured with ^{11}C-Pyruvate and ^{18}F-FDG in cultured neurons with (FCCP group: FCCP) or without (Vehicle group: Cont) FCCP. Each time (min) indicates the period of FCCP application. Note that, following FCCP application, an inducer of mitochondrial dysfunction, ^{11}C-Pyruvate radioactivity decreased, while ^{18}F-FDG radioactivity increased.

3. ^{11}C-pyruvate detected mitochondrial dysfunction induced by intrastriatal injection of 3-NP, a mitochondrial toxin, in rats.

We also studied the feasibility of ^{11}C-pyruvate using in vivo rat model of excitotoxicity. To induce excitotoxic lesion, 3-NP (1 M, 3 μl), a succinate dehydrogenase inhibitor, was microinjected into the striatum in anesthetized rats. Seven days later, rats were received with intravenous injection of ^{11}C-pyruvate (30 MBq, 0.3 ml), decapitated, and brains removed and sectioned into slices. Then, the radioactivity of the tracers in the slices were measured using the imaging plate, and the same slices were stained with 2,3,5-triphenyltetrazolium hydrochloride (TTC) dissolved in physiological saline (2%) to identify the lesions (**Fig. 5**).

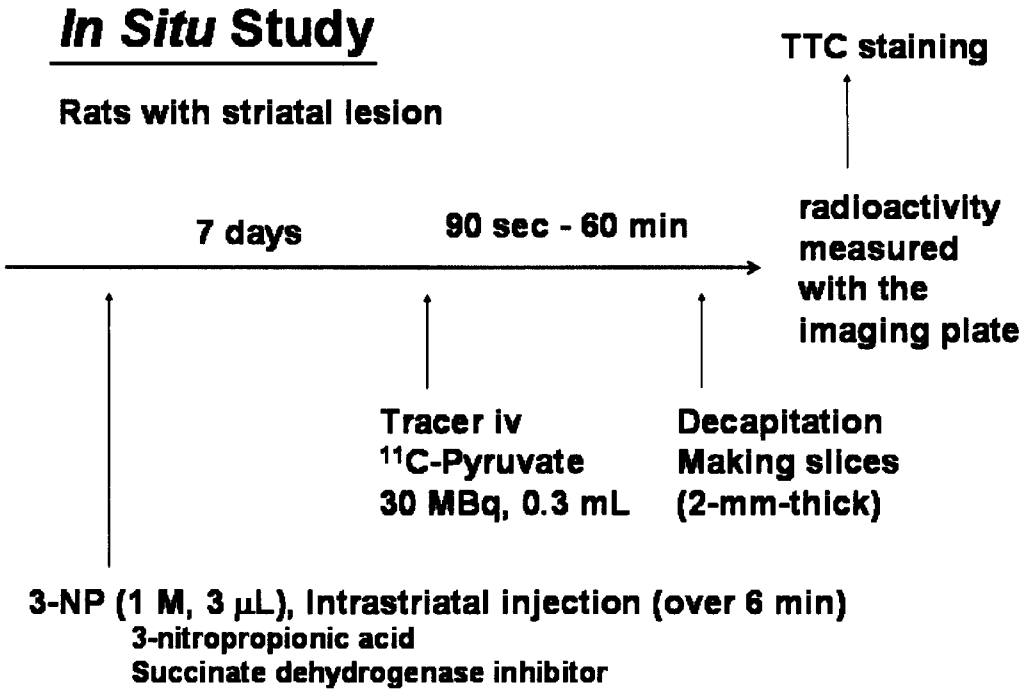

Fig. 5
Protocol for the *in vivo* study using rats received with intrastriatal injection of 3-NP. Excitotoxic lesion with mitochondrial dysfunction in the striatum was induced by intrastiratal microinjection of 3-NP, a succinate dehydrogenase inhibitor, in rats. One week later rates were subjected to the experiment..

The intrastriatal microinjection of 3-NP clearly induced the lesions and decreased the radioactivity of ^{11}C-pyruvate in the lesions (**Fig. 6**). Following the injection of tracer, during the first 90 sec, the tracer radioactivity in the slices, depended upon cerebral blood flow, increased rapidly, and then the activity stayed at the steady level until 60 min. At 10-20 min after injection, the radioactivity was significantly lower in the lesion (**Fig, 7, 8**). Therefore, the results suggested that ^{11}C-pyruvate can be used as a PET tracer to detect the mitochondrial dysfunction when PET scan is performed 10-20 min after the intravenous injection.

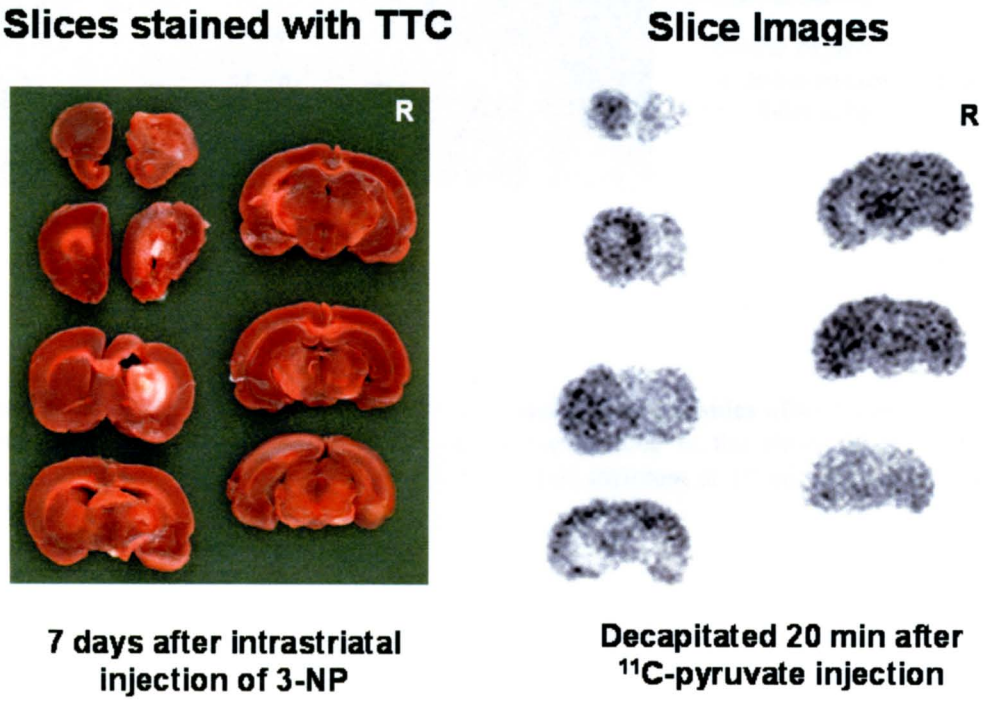

Fig. 6
Representative brain slices stained with TTC and images of ^{11}C-Pyruvate radioactivity measured with an imaging plate. Note that the areas stained red with TTC were viable, and white areas were lesion induced by microinjection of 3-NP. In the images of ^{11}C-Pyruvate, lower radioactivity was noticed in the lesions. TTC; 2,3,5-triphenyltetrazolium hydrochloride.

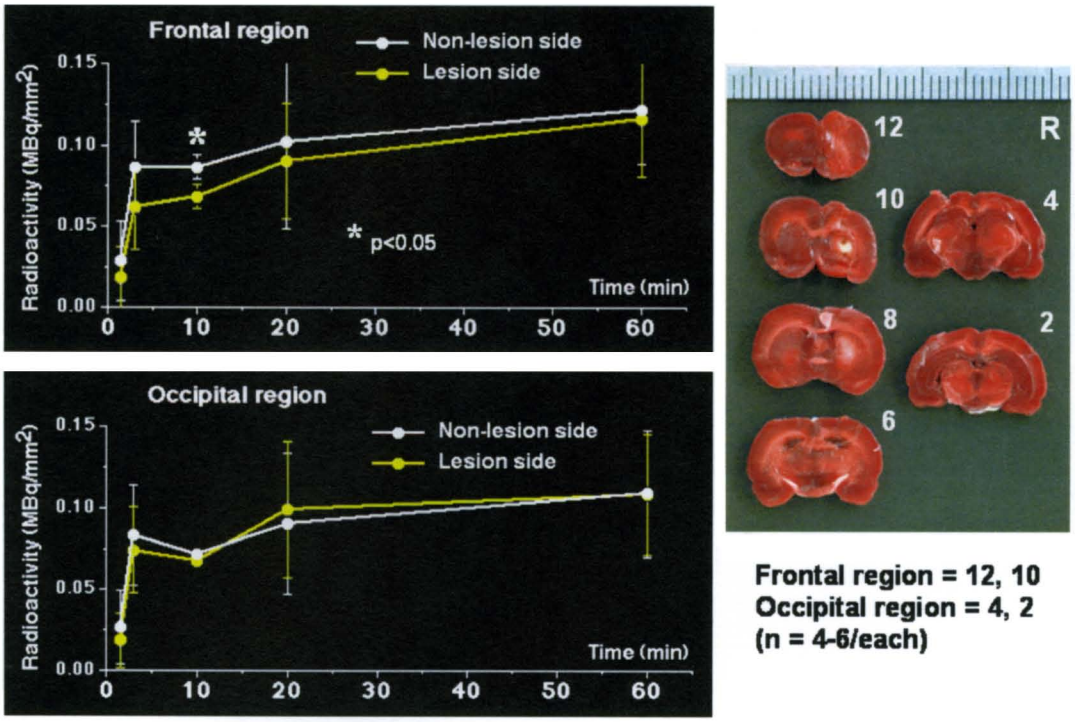

Fig. 7

Change in ^{11}C-Pyruvate radioactivity in the lesion and non-lesion sides after tracer injection in rats. Radioactivity of the frontal region represents mean value in the slices 10 and 12, while occipital region represents in the slice 2 and 4. * $p < 0.05$ different at 10 min between lesion side and non-lesion side (ANOVA).

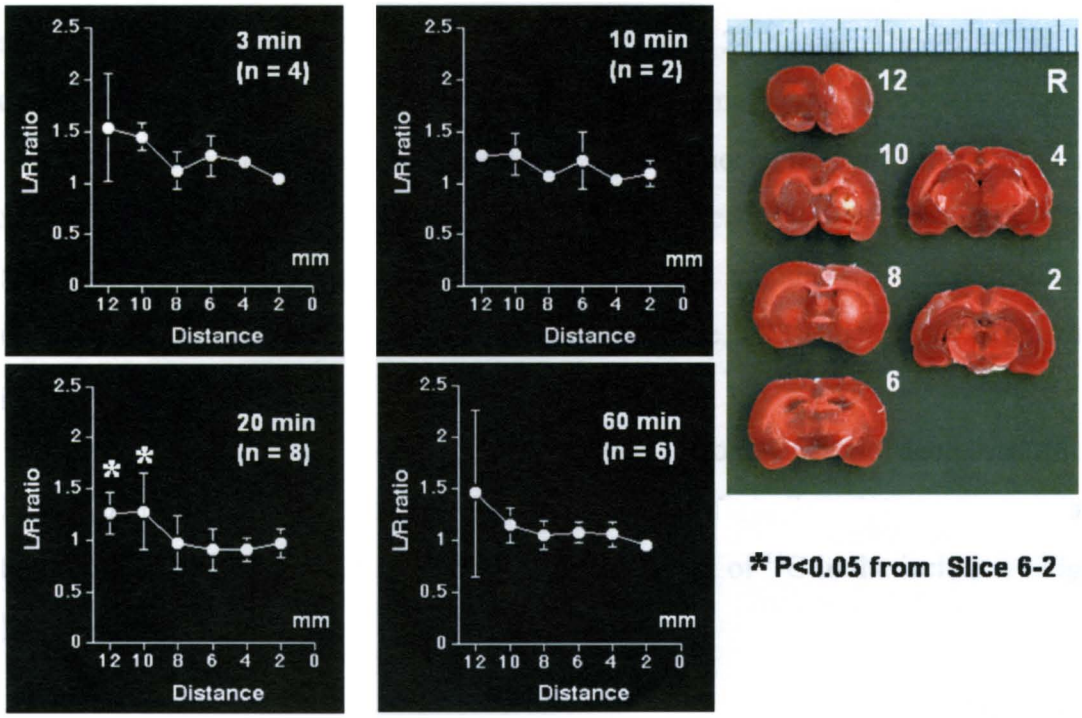

Fig. 8

Distribution of L/R ratio in ^{11}C-Pyruvate radioacitivity following tracer administration in rats. The numbers of "Distance" in the graph represent slice numbers. The L/R ratio > 1 means the radioactivity in the left hemisphere, that is, non-lesion side, was higher that that in the right (lesion side). Note that, at 20 min after tracer injection, in the slice 12 and 10, L/R ratio significantly increased as compare with that in slice 6-2 (occipital region), indicating that the lesion side in the frontal region (lesion area) showed lower radioactivity. * $p < 0.05$ different from the mean value of L/R ratio in the slice 6-2 (ANOVA).

4. ^{11}C-pyruvate detected lesions induced by transient focal ischemia in monkeys.

In one monkey among the three, 3 h after MCA occlusion, reperfusion was very poor. It needed 4 h 10 min to recover CBF, but the animal died 24 h later. Therefore, one was excluded, and data were analyzed from two different animals.

MCA occlusion decreased CBF and $CMRO_2$, and increased OEF. Reperfusion produced hyperperfusion, i.e. CBF increase, and decreased OEF. The $CMRO_2$ was partially recovered by reperfusion. One week and one month after MCA occlusion, while CBF returned to the value of non-ischemic side, $CMRO_2$ and OEF remained slightly lower level (**Fig. 9**).

Eight days and one month after MCA occlusion, CMRglc decreased in the ischemic area. The PET images of ^{11}C-pyruvate showed similar pictures as compared with those of ^{18}F-FDG, showing decreased accumulation of ^{11}C in the ischemic (**Fig. 9, 10**).

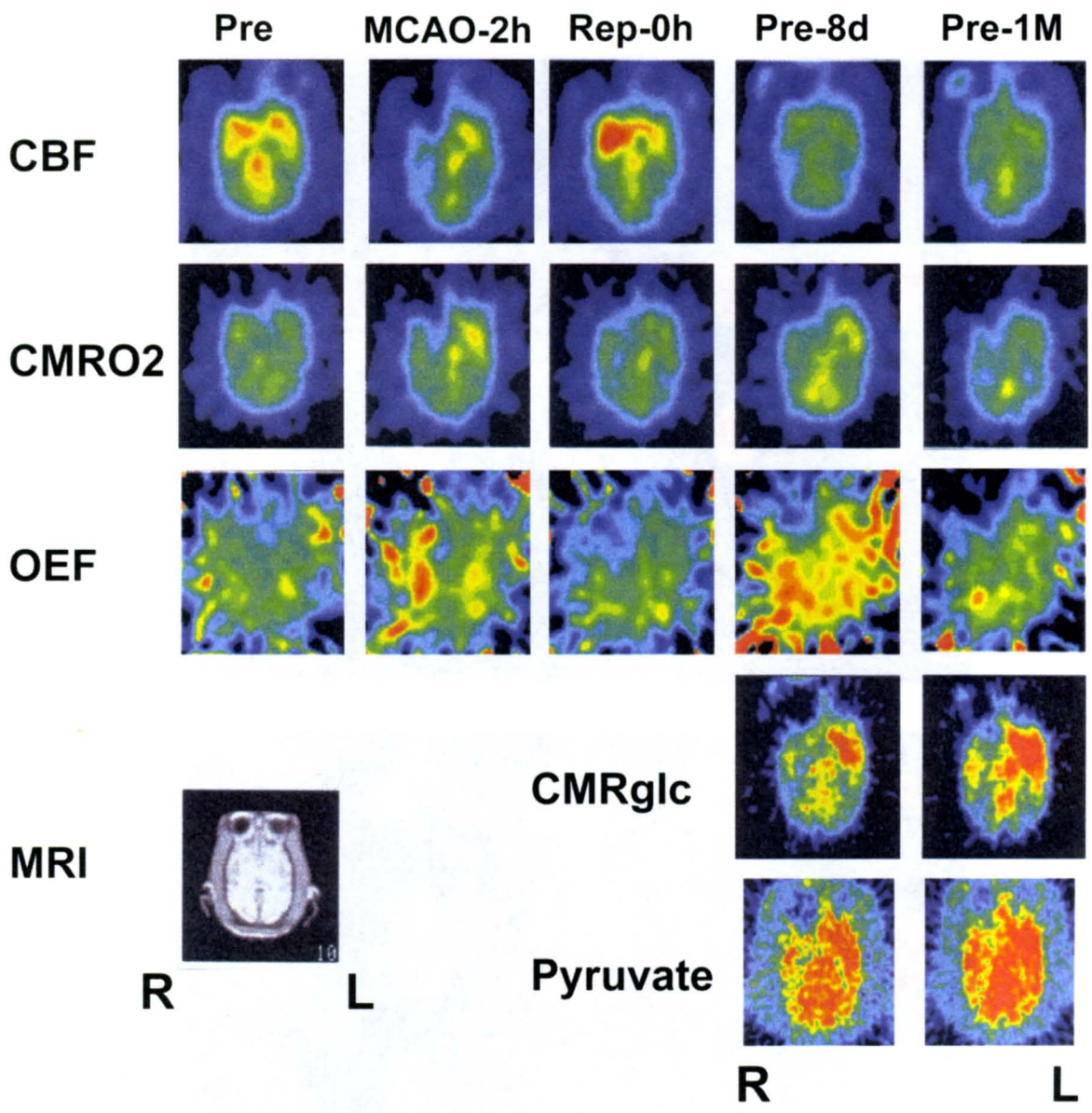

Fig. 9

Representative serial PET images before and after transient MCA occlusion. For CBF, $CMRO_2$, and OEF, PET san was performed before (Pre) and 2 h (MCAO-2h) after MCA occlusion, immediately after reperfusion (Rep-0h), and 8 days (Rep-8d) and 1 month (Rep-1M) after repersuion. For CMRglc and Pyruvate, PET scan was performed 8 days (Rep-8d) and 1 month (Rep-1M) after repersuion. MRI shows the same level in which PET scan is presented. R indicated right hemisphere, i.e. lesion side.

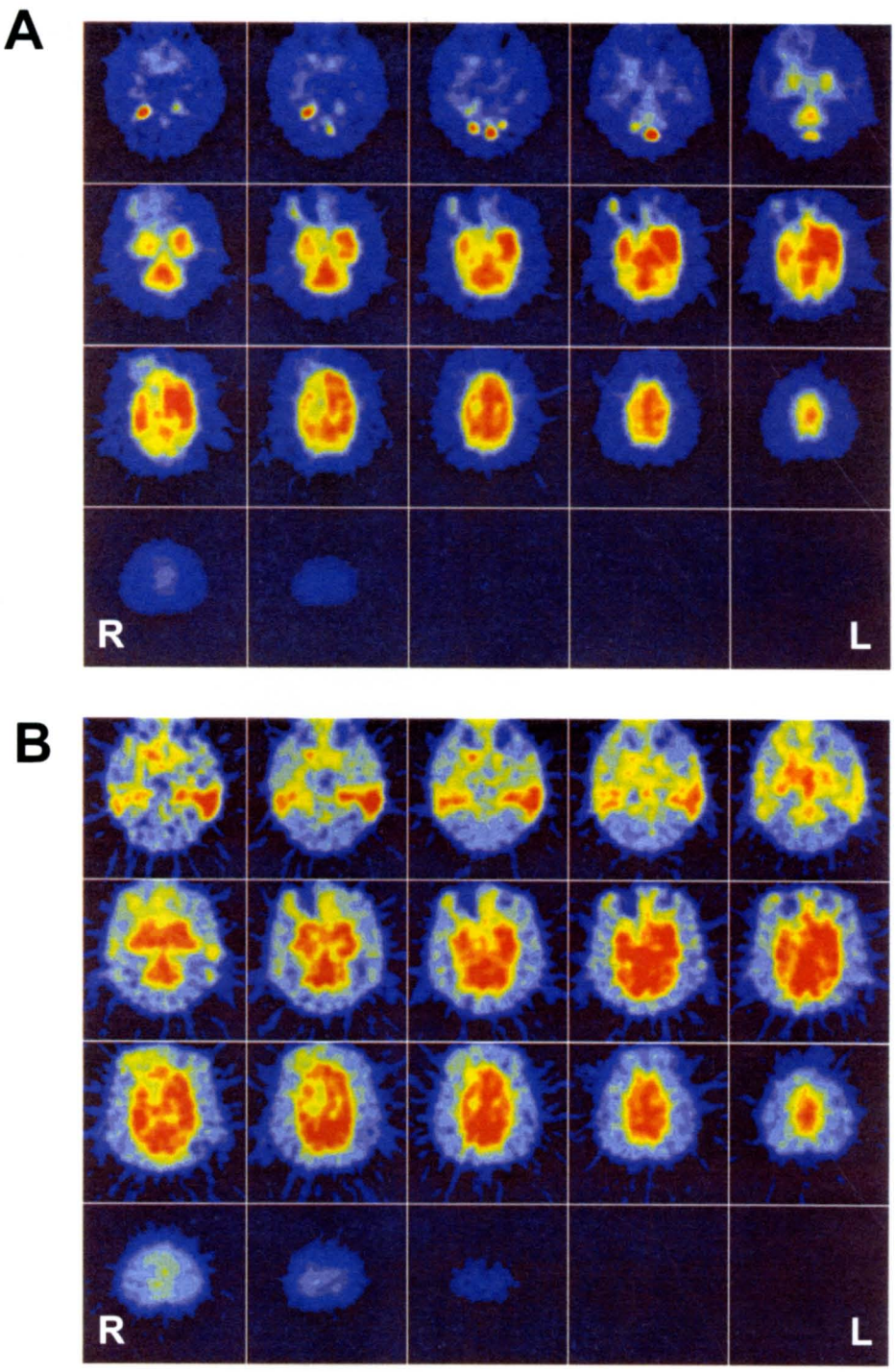

Fig. 10
PET images of ^{18}F-FDG ($CMRO_2$) (A) and ^{11}C-Pyruvate (B). PET scan was performed 8 days after reperfusion. CMRglc and ^{11}C-Pyruvate accumulation decreased in the lesion induced ischemia-reperfusion.

5.Histopathological analysis of transient focal ischemia in monkeys.

Three hours-MCA occlusion and reperfusion produced necrotic damage in the cortex and the basal ganglia one month later (data not shown). Since the necrotic tissue was already absorbed when brain was removed and fixed, the lesion volume was not determined.

Discussion

1. Culture Study

A mitochondrial uncoupler, FCCP impaired mitochondrial membrane potential and induced neuronal death in the cultured hippocampal neurons. During this period FCCP significantly decreased ^{11}C-pyruvate radioactivity and increased ^{18}F-FDG radioactivity. The results indicate that the mitochondrial dysfunction induced by FCCP decreased the turnover rate of pyruvate, and enhanced the anaerobic glycolysis of the neurons. ^{11}C-pyruvate can be useful for analyzing mitochondrial function *in vitro*.

In the cell, glucose is metabolized into pyruvate. Pyruvate is utilized through Citric Acid Cycle in oxidative phosphorylation in the mitochondira (**Fig. 11**).

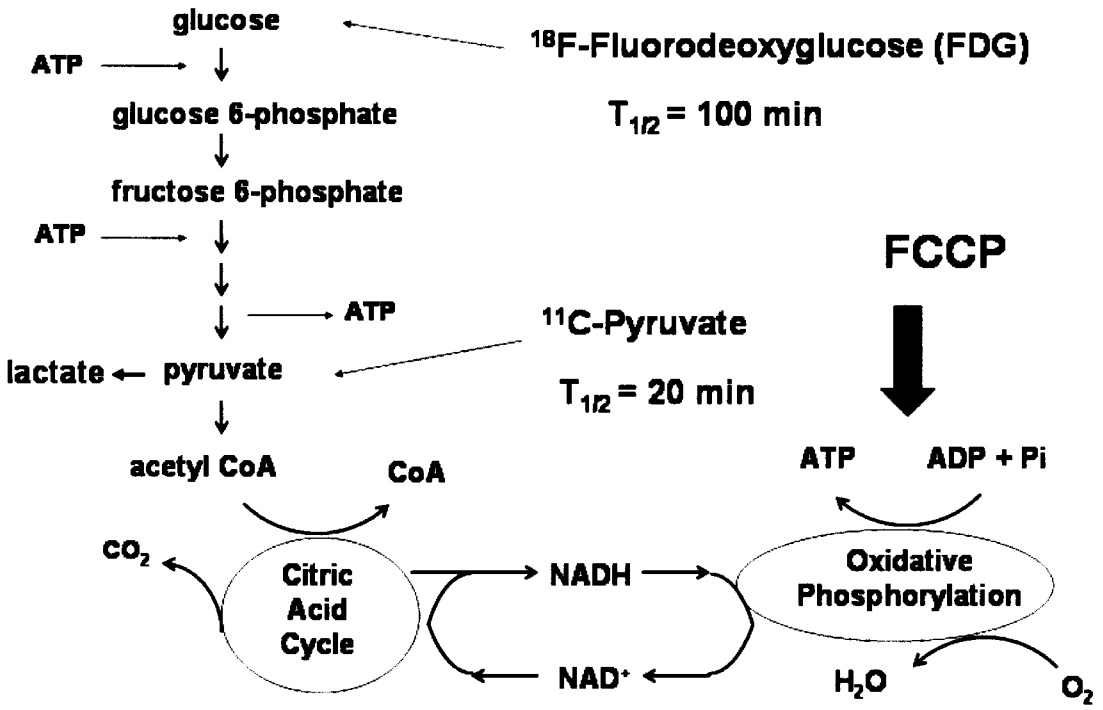

Fig. 11
Metabolism of glucose and pyruvate, oxidative phosphorylation in mitochondira.

While using FDG glucose utilization can be analyzed, it is not a direct measurement in the metabolism of mitochondria. On the contrary, pyruvate is

metabolized downstream. Therefore, ^{11}C-pyruvate may more directly analyze the mitochondrial energy metabolism. FCCP impairs mitochondrial oxidative phosphorylation and mitochondrial membrane potential. In this case, to compensate the aerobic energy failure, glucose is utilized in anaerobic glycolysis in agreement with our results using ^{18}F-FDG, indicating increase in glucose utilization. In the same model of *in vitro* mitochondrial dysfunction, radioactivity of ^{11}C-pyruvate decreased. Our results in vitro model suggest that: 1) ^{11}C-pyruvate can detect the reduction in mitochondrial function; and 2) ^{11}C-pyruvate may be useful to examine the mitochondrial function in the different manner from that used by ^{18}F-FDG.

2. *In Vivo* Rat Study

Intrastriatal injection of 3-NP induces excitotoxicity and the lesion in the striatum, which is called as Huntington's animal model (Koutouzis et al., 1994). Using this model, in the present study, we examined the feasibility of ^{11}C-pyruvate as a PET tracer to detect the mitochondrial dysfunction. The results showed that ^{11}C-pyruvate was detected in the non-affected, intact brain following intravenous injection. After 90 sec the tracer reached steady level, and, 10-20 min later, significant reduction in radioactivity was noticed in the lesion. The *in vivo* study suggests that: 1) ^{11}C-pyruvate can be useful for detecting the mitochondrial dysfunction in the animal model; and 2) ^{11}C-pyruvate can be used as a PET tracer as follows: a) ^{11}C-pyruvate intravenously injected; b) 10–20 min after injection, PET scan performed and PET mages analyzed.

3. Ischemia Study in Monkey

We employed a monkey, a nonhuman primate, and studied the possibility of ^{11}C-pyruvate as a PET tracer for detecting mitochondrial dysfunction in ischemic lesion, a more popular disease. In our results, transient MCA occlusion produced comparable lesion from view point of CBF, $CNRO_2$, and OEF as compared with the previous study (Takamatsu et al, 2001), indicating that MCA occlusion in the present study was suitable for inducing ischemic lesion. In agreement with our study in rat model PET images of ^{11}C-pyruvate showed the reduction in radioactivity in the lesion following reperfusion. Since the CBF did not change in this period, it is not likely that the

reduction in ^{11}C-pyruvate radioactivity was not caused by decreased CBF. The reduction of ^{11}C-pyruvate accumulation must be due to reduction in $CNRO_2$, and OEF. This may be a reflection of aerobic energy metabolism, i.e. mitochondrial function. While the results in our culture study, the accumulation of ^{11}C-pyruvate differed from that of ^{18}F-FDG following an application of FCCP, the PET images of ^{11}C-pyruvate was basically similar to those of ^{18}F-FDG in the study of monkey. Although the reason for the discrepancy remains unclear at present, the stages after ischemia-reperfusion may differ the results of pyruvate and glucose. The further study should be required to reveal the precise metabolism of ^{11}C-pyruvate following ischemia-reperfusion.

A further implication of this study should be considered. To detect the hyperfunction of energy production, ^{18}F-FDG is generally used. In the brain, however, it is difficult to detect the higher function in the metabolism of brain tumor using ^{18}F-FDG, since the basal value of ^{18}F-FDG in the brain is high. The basal accumulation of ^{11}C-pyruvate was lower as compared with that of ^{18}F-FDG in our study. Therefore, if the patterns of ^{11}C-pyruvate are similar to those of ^{18}F-FDG, ^{11}C-pyruvate may be available as a PET tracer to detect malignant brain tumor in the brain.

4. Conclusion

As a PET tracer, ^{11}C-pyruvate is valuable to detect the *in vitro* mitochondrial dysfunction in cultured neurons, the *in situ* excitotoxic lesion in rat, and the lesion induced by ischemia-reperfusion in monkey.

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