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Uptake of radioactive octanoate in astrocytoma cells: Basic studies for application of [^{11}C]octanoate as a PET tracer

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Fatty acids are taken up and metabolized in the brain. *In vitro* uptake experiments on astrocytoma cells were carried out to assess the potential use of [1-^{11}C]octanoate as a positron emission tomography (PET) tracer for astroglial functions. Uptake of [1-^{14}C]octanoate increased in a time-dependent fashion until 60 min after application. The uptake of [1-^{11}C]octanoate showed similar results to that of [1-^{14}C]octanoate until 10 min. As for medium pH, [1-^{14}C]octanoate uptake increased gradually with the decrease in pH. We also examined the effects of glutamate, glucose deprivation and hypoxia on the uptake of octanoate and found that these conditions did not bring about any change in the extent of [1-^{14}C]octanoate uptake. These results show that the octanoate uptake was not influenced by any of several pathological conditions. When the number of astrocytes increases in the area of hypoglycemia or hypoxia near a brain lesion, the amount of octanoate uptake also increases, so this indicates the possibility that ^{11}C-octanoate will detect a brain lesion.

Key words: astrocytoma cell culture, ^{11}C-octanoate, ^{14}C-octanoate, pathological conditions

INTRODUCTION

MANY PET STUDIES have recently been performed to investigate brain function and have contributed to accurate diagnoses of several neurological diseases. Fatty acid derivatives labeled with positron emitting nuclides were synthesized and investigated in biodistribution studies at some laboratories.[1-3] Long chain fatty acids are taken up into the brain and are incorporated into brain tissue lipids.[4] Octanoate, an 8-carbon monocarboxylic saturated fatty acid, is not incorporated into lipids but rather converted to glutamine through the tricarboxylic acid cycle following β-oxidation.[3,5] Octanoate was reported to be more rapidly taken up in brain than other mono-, di- or tricarboxylic saturated fatty acids.[6] Studies of fatty acid utilization in primary cultures of neurons, oligodendrocytes, and astrocytes clearly indicated that only astrocytes were able to utilize octanoate.[7,8] We therefore speculate that octanoate may be used as a potential specific marker for astrocytes. In the damaged brain, reactive astrocytosis appears near and around the lesions,[9,10] resulting in higher density of reactive astrocytes near and around the lesions than in other areas. In these respects, lesions are represented as positive images which are useful in clinical diagnosis. In this study, we investigated the uptake of octanoate in astrocytoma cells under various physiological and pathological conditions in order to provide a basis for the application of [1-^{11}C]octanoate as a PET tracer for detecting brain lesions through astroglial functions.

MATERIALS AND METHODS

[1-^{11}C]Octanoate was synthesized by a Grignard reaction of $^{11}CO_2$ with heptyl magnesium bromide[11] in an automated synthesis apparatus (CUPID, Sumitomo Heavy Industries Co., Ltd.). Briefly, $^{11}CO_2$ was produced in an ultracompact cyclotron (CYPRIS HM-18, Sumitomo Heavy Industries Co., Ltd.) by the $^{14}N(p,\alpha)^{11}C$ reaction in a N_2 gas target. The ^{11}C-labeled CO_2 was trapped in a stainless coil dipped in liquid argon and then introduced into a reactor, reacted with the Grignard reagent, and the

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product was hydrolyzed with hydrochloric acid. [1-^{11}C]Octanoate was then purified by high performance liquid chromatography (HPLC) on a reverse-phase column (Cosmosil $5C_{18}$-AR, 250 mm × 10 mm i.d., Nacalai Tesque, Co., Ltd.) with CH_3CN/0.012 M HCl (50/50, vol/vol). The eluate was collected in a flask (100 m*l*) containing 7% $NaHCO_3$ aq (1 m*l*) of a rotary evaporator and concentrated under reduced pressure at 80°C to remove the organic solvent. The radiochemical purity of the [1-^{11}C]octanoate obtained was found to be > 99% by means of HPLC on a reverse-phase column (YMC PAC ODS-AQ, 250 mm × 4.6 mm i.d., YMC Co., Ltd.) with CH_3CN/0.015 M HCl (60/40, vol/vol), and the specific activity was > 100 GBq/mmol at the end of formation.

[1-^{14}C]Octanoate (radiochemical purity: 99%, specific activity: 2.0 GBq/mmol) was purchased from NEN Research Products (Du Pont Co., Ltd.).

Cell culture

The human astrocytoma cell line U-373MG was obtained from American Type Culture Collection at passage 181 and cultured in Eagle's minimum essential medium (EMEM) with 10% fetal calf serum (FCS). Cells were grown in collagen-coated tissue culture dishes and were used between passages 187 and 192. For uptake experiments, cells were removed from the dishes by trypsinization and were plated onto collagen-coated 24-well plates 3 to 4 days before use. All cells were cultured at 37°C in a 95%/5% (vol/vol) mixture of air and CO_2 humidified atmosphere.

Astrocytic characterization

mRNAs of human astrocytoma were obtained from U-373MG by Micro-Fast Track (Invitrogen) with some modifications. The reverse transcription reactions were performed with 0.2 μg of mRNA. A GeneAmp RNA PCR kit (Perkin-Elmer) was used to amplify the cDNAs transcribed from mRNAs. Primers for polymerase chain reaction (PCR) amplification of glial fibrillary acidic protein (GFAP) cDNA were 5′ AGGAGAACCGGATCACCATT 3′ (sense) and 5′ CCTCATTCTAACGCAAGCTG 3′ (antisense), encompassing bases 1121–1140 and 1567–1586, respectively.[12] Those of glutamine synthetase (GS) were 5′ AAATAAAGGCATCAAGCAGG 3′ (sense) and 5′ TTCACAAGGTCCAATCTGAA 3′ (antisense), encompassing bases 146–165 and 730–749, respectively.[13] The amplified products were 466 bp for GFAP cDNA and 604 bp for GS cDNA. AmpliTaq polymerases (Perkin-Elmer) and a Thermal Cycler were used to amplify cDNAs in a 100 μ*l* reaction volume with 30 cycles consisting of a 1-min denaturation period at 94°C, a 2-min annealing period at 55°C, and a 3-min period at 72°C for polymerization of the annealed primers. Following amplification, the reaction products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide using λ-Hind III as a standard.

[1-^{11}C] and [1-^{14}C]Octanoate uptake studies

Before the uptake experiment, the cultures were incubated in Hanks' balanced salt solution (HBSS, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5 mM KCl, 0.3 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$) containing 20 mM Hepes instead of $NaHCO_3$ (Hanks-Hepes solution) for 1 hr. The pH of the medium was adjusted to 7.2, 7.4 and 7.6 by 10 M NaOH. Uptake of radiotracer was initiated at the time of changing the culture medium to the radiotracer-containing medium. The concentration of ^{11}C-octanoate containing Hanks-Hepes solution was 12.0–13.9 MBq/m*l*. The ^{14}C-octanoate ethanol solution, commercially obtained, was evaporated at 4°C and the residue was dissolved in Hanks-Hepes solution. The concentration of radioactivity was 3.7×10^{-3} MBq/m*l*. Cells were rapidly washed 4 times at each time point after exposure to the radiotracer-containing medium, with ice-cold phosphate-buffered saline to terminate the octanoate uptake. The cells were then solubilized with 0.1 M NaOH and the radioactivity was measured in a well-type (1480 WIZARD™3″, Wallac Co., Ltd.) and a liquid (1215 RACKDETA, LKB) scintillation counter for ^{11}C and ^{14}C, respectively. ULTIMA GOLD MV (PACKARD JAPAN K.K.) was used as the liquid scintillator to detect ^{14}C. An aliquot of radiotracer-containing medium was also measured to obtain a total count. Octanoate uptake was expressed as uptake count per total count. In the case of ^{11}C, the calculations were made after decay correction. Protein content was measured by BCA Protein Assay Reagent (Pierce Chemical Co.) with bovine serum albumin as a standard. All results are expressed as the mean ± SE from three or six experiments of duplicate or triplicate assays. The numbers of experiments are shown in the figure legend.

The effects of glutamate exposure, glucose deprivation and hypoxia on [1-^{14}C]octanoate uptake were studied. To assess the effects of glutamate, cells were pre-incubated with 0.5 and 1 mM glutamate for 21 hr prior to the uptake experiments. To evaluate the effects of hypoglycemia, cells were pre-incubated with glucose-deprived HBSS for 24 hr before the uptake experiments. For hypoxia, cultures were placed in a modular incubator (Billups-Rothenberg Inc.) which was sealed tightly after being purged for 15 min with a 95%/5% (vol/vol) mixture of N_2 and CO_2 and were incubated at 37°C for a further 18, 24 and 48 hr.[14,15] ^{14}C-Octanoate uptake experiments and protein assays were then performed by the procedures described above.

RESULTS AND DISCUSSION

The middle chain fatty acid, octanoate, is taken up and metabolized in astrocytes but not in neurons or oligodendrocytes.[7] Reactive astrocytes proliferate at the site of a brain lesion.[9,10] So we believe that octanoate may serve as a PET tracer in detecting brain lesions through astroglial

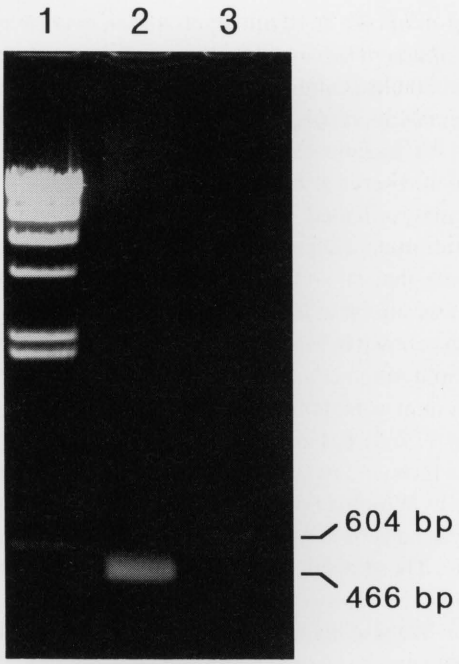

Fig. 1 Examination of U-373MG cells for the expression of GFAP and GS mRNA. mRNA was extracted from U-373MG cells, amplified by RT-PCR, and the amplified products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Lane 1: λ-Hind III, Lanes 2 and 3: amplified PCR products obtained using primers specific for GFAP and GS, respectively.

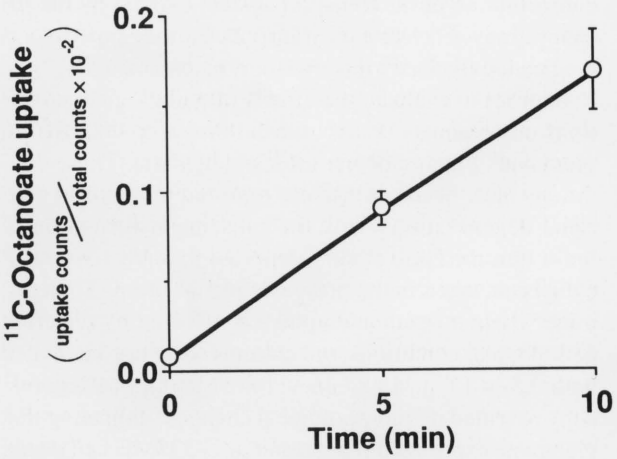

Fig. 2 Uptake of [1-^{11}C]octanoate in astrocytoma cells. The uptake is expressed as a ratio of uptake radioactivities and total radioactivities in the well. The values are given as means ± SE from n = 3.

functions. We chose to use the astrocytically-derived U-373MG cell line as a first step because of the relative ease with which astrocytoma cells can be cultured and maintained. It is well known that GFAP and GS are specific marker proteins for astrocytes.[9,10,16] Reverse transcriptase (RT)-PCR procedures were therefore performed to confirm the expression of GFAP and GS mRNAs in U-373MG cells. Shown in Fig. 1 are the bands obtained from

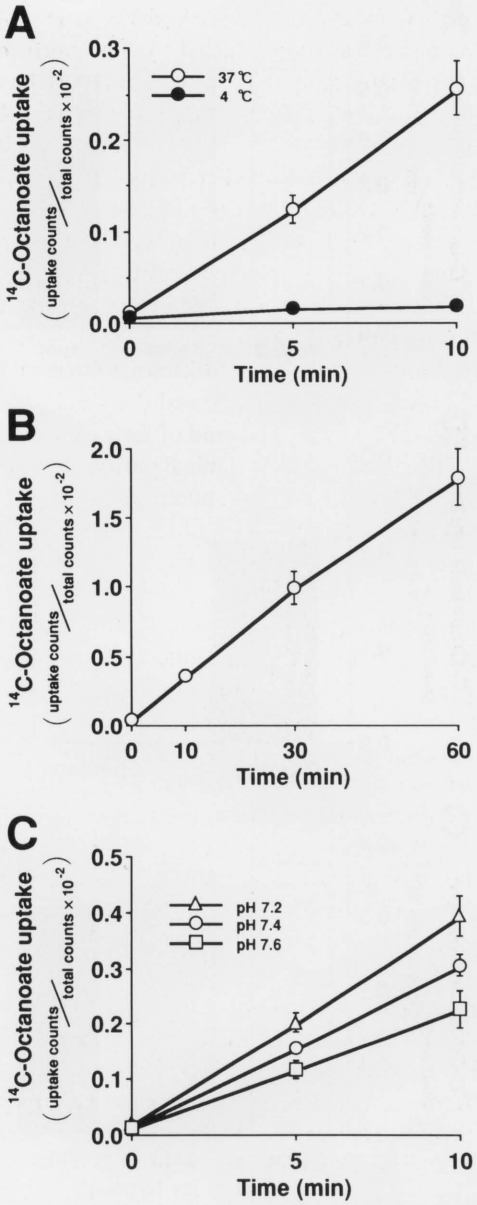

Fig. 3 Uptake of [1-^{14}C]octanoate in astrocytoma cells. A: Effect of medium temperature on [1-^{14}C]octanoate uptake. Closed; 4°C and open; 37°C, B: Time course of [1-^{14}C]octanoate uptake, C: Effect of medium pH on [1-^{14}C]octanoate uptake. Triangle, circle and square indicate the medium pH of 7.2, 7.4 and 7.6, respectively. The values are given as means ± SE from n = 3. Only in pH 7.4 group, it is given from n = 6.

RT-PCR of U-373MG cells mRNA. As expected from their nucleotide sequence, the fragments sizes are 466 bp (lane 2) and 604 bp (lane 3), corresponding to GFAP and GS mRNA, respectively. These data clearly show that GFAP and GS mRNA are expressed in U-373MG cells.

The purpose of our study was to assess the potential use of [1-^{11}C]octanoate as a diagnostic PET tracer.[3] The short half life of the ^{11}C isotope (20.4 min) allowed for only short experiments so that the uptake of [1-^{11}C]- and [1-^{14}C]octanoate was examined to compare the results.

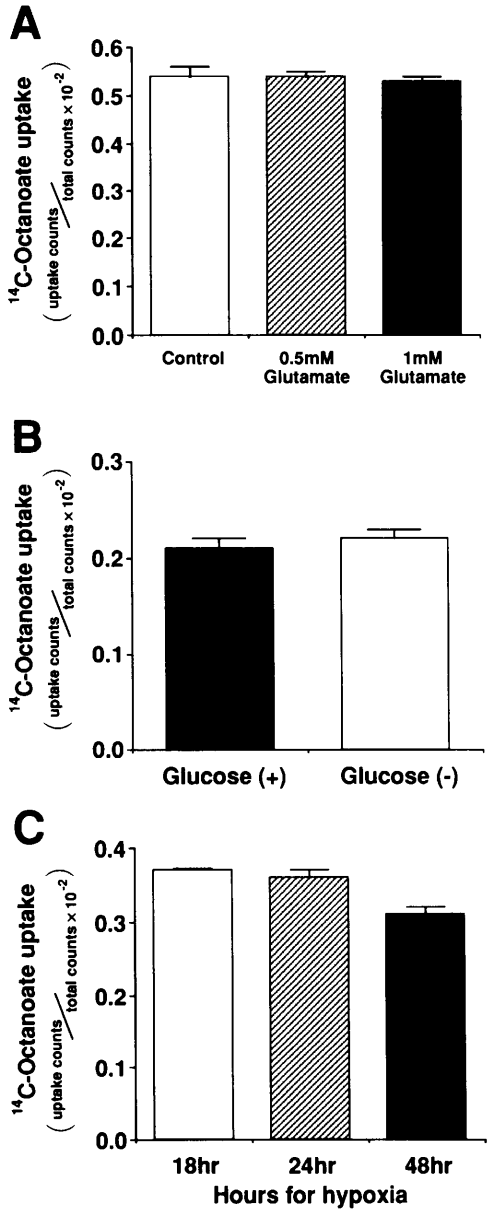

Fig. 4 Uptake of [1-^{14}C]octanoate under pathological conditions. A: Effect of glutamate on [1-^{14}C]octanoate uptake. Open column shows the uptake without glutamate, hatched and closed columns show the uptake with 0.5 and 1 mM glutamate, respectively. Astrocytoma cells were incubated in the presence and absence of glutamate for 21 hr before the experiment. B: Effect of glucose on [1-^{14}C]octanoate uptake. The cells were incubated in HBSS in the presence and absence of glucose for 24 hr before the experiment. C: Effect of hypoxia on [1-^{14}C]octanoate uptake. The cells were incubated in 95% N_2/5% CO_2-filled, tight-sealed chamber for 18, 24 and 48 hr before the experiments. The values are given as means ± SE from n = 3.

Figure 2 shows the results of the [1-^{11}C]octanoate uptake experiment. The uptake of octanoate increased gradually for 10 min. Using a similar experimental paradigm to that with [1-^{11}C]octanoate, we then studied [1-^{14}C]octanoate uptake. As shown in Figs. 2 and 3A, the time course of uptake of [^{11}C]- and [^{14}C]octanoate was similar, with the amount of uptake at 10 min approximately double that at 5 min, although the specific radioactivities were different (100–1000 GBq/mmol and 2.0 GBq/mmol for [^{11}C]- and [^{14}C]octanoate, respectively). Based on these results, we used [1-^{14}C]octanoate to determine whether [1-^{11}C]octanoate could serve as a useful tracer.

As also indicated in Fig. 3A, octanoate uptake was temperature-dependent with uptake at 4°C approximately one tenth that at 37°C. The protein content in the 4°C group was similar to that in the 37°C group (57.9 ± 5.3 μg/well). As shown in Fig. 3B, [1-^{14}C]octanoate was taken up into astrocytoma cells in a time-dependent manner. Protein content was determined to be 54.1 ± 3.1 μg/well. Spector (1988) has reported that octanoate is taken up into the brain *in vivo*, in large part by a specific transport system.[17] We observed that the uptake of octanoate in astrocytoma cells at 4°C was significantly lower than that at 37°C. These results agree with a specific transporter-mediated mechanism of octanoate uptake, as proposed by Spector. We also investigated the effect of medium pH on octanoate uptake. As shown in Fig. 3C, octanoate uptake was decreased with increasing pH with the highest uptake observed at pH 7.2. The protein content in this series of experiments was 62.1 ± 2.7 μg/well. The cause of this influence remains to be clarified. With the decrease in pH, the portion of undissociated octanoate increases, and this may lead to the enhancement of passive transportation of octanoate. Another possibility is that some alteration in conformation of the transport system evoked by the pH change may accelerate the transport. Further experiments are needed to clarify this transport mechanism.

In order to evaluate the effects of pathological conditions on octanoate uptake, the cultures were exposed to glutamate, glucose deprivation and hypoxia. These conditions have been extensively reported to result in neuronal degeneration[14] with the subsequent formation of brain lesions. Petio et al.[9,10] reported that reactive astrocytosis occurred in the postischemic rat brain. To determine whether octanoate uptake is affected by different pathological conditions, the cultures were pre-incubated with 0.5 or 1.0 mM glutamate for 21 hr. Optical microscopy revealed no morphological changes, indicating that glutamate exposure did not result in U-373MG cell death. Furthermore, the protein content was also unchanged, giving values of 74.0 ± 1.7 μg/well for control, 74.9 ± 1.2 μg/well for 0.5 mM glutamate and 77.9 ± 1.4 μg/well for 1.0 mM glutamate. As shown in Fig. 4A, octanoate uptake was also unaffected by glutamate. Glucose deprivation (Fig. 4B) and hypoxia (Fig. 4C) also exerted no effect on [1-^{14}C]octanoate uptake although it was slightly reduced after 48 hr of hypoxia. Cell morphology and protein content (63.8 ± 2.0 μg/well) were similar in all hypoxia groups. We have shown that octanoate uptake was not affected by these conditions (Fig. 4). These pathological conditions were easily reproduced in cultured cell levels. The concentrations of glutamate used were high enough

to cause neuronal cell death.[18] The octanoate uptakes in glucose deprivation and in hypoxia were also investigated in cultured cell systems simulating brain ischemia *in vivo*. The present data suggest that U-373MG cells are resistant to glutamate exposure, glucose deprivation and hypoxia.

In conclusion, the present study showed that octanoate is taken up in astrocytic cells without being affected by various pathological conditions. The present results therefore suggest that the uptake of octanoate will increase at sites near to and surrounding brain lesions, if only the population of the astrocyte is increased there, without being influenced by other factors such as prevailing glucose deprivation and hypoxia. Furthermore, it may be possible with the use of [1-^{11}C]octanoate to detect brain lesions as positive PET images. Further studies are required to confirm this possibility by using primary glial cultures and *in vivo* studies.

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