Incorporation of [U-^{14}C]palmitate into rat brain: effect of an inhibitor of β-oxidation

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Abstract We examined the effect of a clinically therapeutic dose of methyl 2-tetradecylglycidate (McN-3716, methyl palmoxirate, MEP) (2.5 mg/kg), an inhibitor of β-oxidation of fatty acids, on incorporation of radiolabeled palmitic acid ([U-^{14}C]PAM) from plasma into brain lipids of awake rats. Four hour pretreatment with 2.5 mg/kg MEP significantly increased the incorporation of [U-^{14}C]PAM into brain lipids and substantially decreased aqueous radiolabeled metabolites in brain that can constitute unwanted background signal when analyzed by quantitative autoradiography. MEP treatment increased the lipid to aqueous background radioactivity from 0.8 to 3.0. Net rate of incorporation, k*, was significantly increased (60%) by MEP and was attributed to incorporation of [U-^{14}C]PAM into phospholipid and triglyceride brain compartments. MEP treatment did not affect the size of the fatty acyl-CoA pool or the distribution of the various molecular acyl-CoA species. These results indicate that MEP, at a dose of 2.5 mg/kg (per os), can be used to increase incorporation of [1-^{11}C]PAM for studying brain lipid metabolism in humans by positron emission tomography (PET).—**Chang, M. C. J., E. Grange, O. Rabin, and J. M. Bell.** Incorporation of [U-^{14}C] palmitate into rat brain: effect of an inhibitor of β-oxidation. *J. Lipid Res.* 1997. **38**: 295–300.

Supplementary key words methyl palmoxirate • palmitate • fatty acids • phospholipids • brain • rat • in vivo imaging • β-oxidation

In our laboratory, we are studying brain functional activity and structural integrity as they relate to rates of formation and turnover of fatty acids in brain phospholipids. To do this, we have developed an in vivo method and model to study brain phospholipid metabolism after the intravenous injection of a radiolabeled long-chain fatty acid. We have used three different fatty acid radiotracers, [1-^{14}C]arachidonate, [1-^{14}C]docosahexaenoate and [9,10-^{3}H]palmitate to pulse-label individual brain lipid compartments, particularly phospholipids, by this method (1–4). These tracers have a selective pattern of incorporation into brain phospholipids and thus may be used as probes to study structural and physiological changes in brain (2, 5–10). Furthermore, autoradiographic and PET studies indicate that intravenously infused ^{11}C-radiolabeled fatty acids can be used to image brain under normal and pathological conditions (2, 5–12) (Chang, et al., unpublished observations). Unlike the unsaturated fatty acids, much of radiolabeled palmitate that enters brain is funneled via β-oxidation in mitochondria into aqueous pools, resulting in high background aqueous radioactivity when analyzed by quantitative autoradiography or PET (3, 12, 13). For autoradiographic studies, this limitation is overcome by using [9, 10-^{3}H]PAM, as 75% of the tritium is converted to [^{3}H]water after oxidation and is eliminated during autoradiographic processing (3). However, to extend the fatty acid method and model to human, with [1-^{11}C] fatty acids and PET, we had to adopt a strategy to reduce background radiation. We approached this problem by using an inhibitor of β-oxidation, methyl palmoxirate (MEP) (McNeil Pharmaceuticals, Spring House, PA), which increases the fraction of plasma radiolabeled palmitate incorporation into brain lipid (13, 14) and reduces aqueous metabolites. The dose of MEP used in these previous animal studies (10 mg/kg) (12–14) and in heart studies with PET (15) has never been used for investigational clinical studies. McNeil Pharmaceutical-sponsored clinical investigations, to evaluate efficacy and safety of MEP as a hypoglycemic agent in diabetics, indicate that the drug was tolerated without side effects in human subjects at an oral dose of approximately 2.5 mg/kg. To evaluate the drug's potential clinical use with the fatty acid method at that dose, this present study was undertaken to determine if and to what extent brain mitochondrial β-oxidation of palmitate could be inhibited. We examined the effect of MEP, at a dose of

Abbreviations: MEP, methyl palmoxirate; PAM, palmitic acid; CoA, coenzyme A; PET, positron emission tomography; PL, phospholipids; MG, monoglycerides; CHOL, cholesterol; DG, diglycerides; FFA, free fatty acids; TG, triglycerides; CE, cholesteryl esters; CAT I, carnitine acyltransferase I; BSA, bovine serum albumin.

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2.5 mg/kg, on incorporation of radiolabeled palmitic acid into brain lipids. The effect of the drug on the brain acyl-CoA pool, the precursor pool for the incorporation of PAM and other long-chain fatty acids into brain phospholipids, was also examined. The results indicate that MEP at a dose of 2.5 mg/kg per os increases incorporation of [U-^{14}C]PAM into brain lipids, reduces radiolabeled aqueous metabolites in brain, does not affect the concentration of the acyl-CoA pool and, therefore, could be used to inhibit oxidation of [1-^{11}C]PAM for PET studies to study brain lipid metabolism.

MATERIALS AND METHODS

Male Fischer 344 rats (260–280 g, Charles River Laboratories, Wilmington, MA) served as experimental animals. The study was conducted according to National Institutes of Health guidelines (Publication No. 80-23) and approved by the NICHD Animal Care and Use Committee. Methyl palmoxirate (MEP, McN-3716) was generously donated by McNeil Pharmaceutical Spring House, PA). [U-^{14}C]PAM (specific activity >500 mCi/mmol) was purchased from NEN (Boston, MA). Bovine serum albumin (BSA), essentially fatty acid free (Fraction V), was purchased from Sigma Chemical Co. (St. Louis, MO). MEP (1.4 mg/ml) was dispersed in tragacanth (0.5%), and administered to rats (2.5 mg/kg) per os after recovery from surgical procedure. [U-^{14}C]PAM was evaporated under N_2 and then solubilized by sonication in 50 mg/ml BSA (5 mM HEPES buffer, pH 7.4) to a concentration of 40 μCi/ml.

Animals were prepared and [U-^{14}C]PAM was infused as previously described (13). The surgical procedure involved implanting of polyethylene catheters into the femoral artery and vein under 1–3% (v/v) halothane anesthesia. The rat was wrapped loosely in a fast setting cast and taped to a wooden block and allowed to recover from anesthesia in a temperature-controlled hood (25°C). Four hours after drug or vehicle treatment, [U-^{14}C]PAM (75 μCi/kg) was infused (iv) over a 5-min period using a constant rate infusion pump (Harvard Apparatus, South Natick, MA). Timed arterial blood samples (100 μl) were collected after beginning fatty acid infusion. At 20 min after the start of infusion, the rat was killed with sodium pentobarbital (60 mg/ml), decapitated, and its brain was excised from the skull. Plasma and brain lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (16). Brain lipid and phospholipid classes were separated by one-dimensional thin-layer chromatography using heptane–diethylether–glacial acetic acid 60:40:2 (v/v) and chloroform–methanol–glacial acetic acid–water 60:50:1:4 as the developing solvent, respectively. Radioactivity in brain lipid and aqueous fractions was quantified by liquid scintillation spectroscopy.

The regional incorporation coefficient (k*) for [U-^{14}C] PAM was calculated as follows (4):

$$k^* = \frac{c^*_{brain}}{\int_0^{20} c^*_{plasma} dt}$$

where k* is in units of ml/sec · g and c^*_{brain} (in units of nCi/g) is brain organic radioactivity at 20 min. c^*_{plasma} (in units of nCi/ml) is plasma fatty acid organic radioactivity and t is time (min) after the start of infusion. For acyl-CoA analysis, rats were killed with a lethal dose of sodium pentobarbital and irradiated by a focused-beam microwave (5.5 kW, 3.0 s) (Cober Electronics, Stanford, CT). Acyl-CoA was extracted and acyl-CoA molecular species were separated according to method of Deutsch et al. (17). All values represent mean ± standard error (SEM) for (n) number of experiments. Statistical significance was determined by unpaired *t*-test (Instat 2.03).

RESULTS AND DISCUSSION

This study investigated the effects of oral administration of MEP (2.5 mg/kg), on the incorporation of [U-^{14}C]PAM from plasma into brain lipids of awake rats. MEP significantly increased incorporation of [U-^{14}C] PAM into brain lipids and decreased aqueous radioactivity (unwanted background signal), without altering the total amount of brain radioactivity (**Fig. 1**). Organic radioactivity in vehicle-treated rats accounted for 46% of total radioactivity and increased to 75% with MEP treatment, thus raising the lipid/aqueous background radioactivity from 0.8 to 3.0. This increase was lower than reported in similar studies when MEP was administered to rats at a dose of 10 mg/kg consistent with a dose effect from 0.7 to 5.7 (13, 14). Elevation in brain lipid radioactivity was reflected in the percent infused lipid radioactivity that was significantly increased from 0.06 ± 0.00% to 0.08 ± 0.00% ($P < 0.01$). These results suggest that MEP at 2.5 mg/kg (p.o.) inhibits brain oxidative metabolism of [U-^{14}C]PAM. The data also suggest that the transfer of palmitate across the blood–brain barrier is not affected by MEP because the amount of total brain radioactivity was not altered by drug treatment. MEP treatment only increased the distribution of palmitate from brain aqueous to brain organic compartments. As MEP is a fatty acid analog, it is likely bound

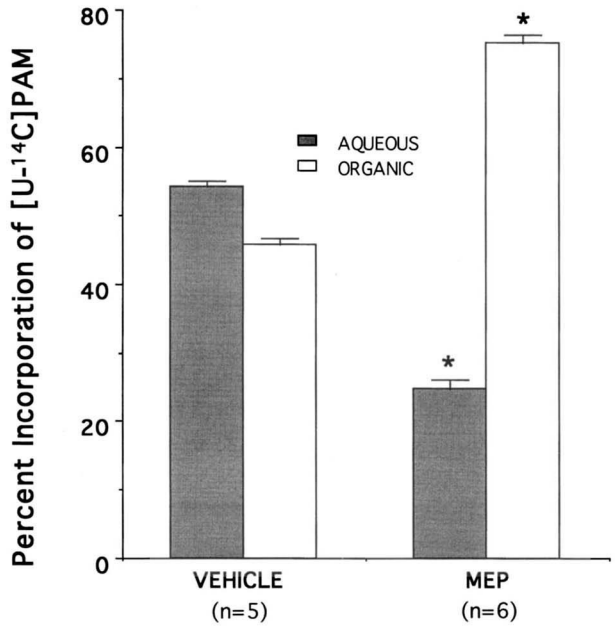

Fig. 1. The effect of MEP (2.5 mg/kg, 4 h pretreatment) on the distribution of [U-^{14}C]palmitate-derived label in brain fractions. Brain extracts, collected after infusion of isotope, were partitioned into aqueous and organic phases. Each bar represents the mean ± SEM. Statistical significance (*$P < 0.001$) was observed between MEP-treated and vehicle.

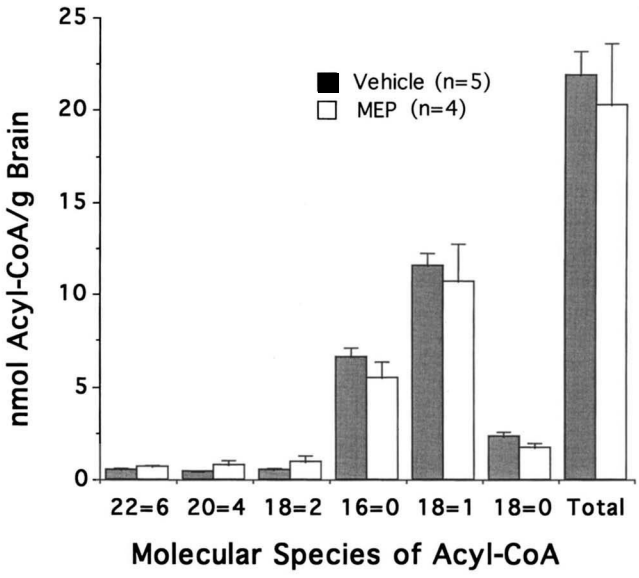

Fig. 2. Comparison of mean concentrations of brain acyl-CoA from rats treated with MEP (2.5 mg/kg, 4 h pretreatment) and vehicle. Each bar represents the mean ± SEM. No statistical significance was observed between MEP-treated and vehicle-treated rats.

to serum albumin and enters brain and can become activated with coenzyme A (CoA) (18, 19). It is this CoA ester of MEP that binds to carnitine acyltransferase I to form a tight complex that inactivates the enzyme that oxidizes long-chain saturated fatty acids (20).

Net rate of incorporation of [U-^{14}C]PAM into brain of rats, k* (Eq. 1), with and without MEP treatment, was $7.0 \times 10^{-5} \pm 0.2 \times 10^{-5}$ and $11.2 \times 10^{-5} \pm 0.8 \times 10^{-5}$ ml/sec · g ($P < 0.01$), respectively. Although the effect of MEP, 2.5 mg/kg, on incorporation of [U-^{14}C]PAM into total brain lipids is consistent with other studies from our laboratory (10 mg/kg) (13, 14), its effect on the distribution of radioactivity into various lipid classes was different (**Table 1**). At this lower dose the percentage of radioactivity in brain phospholipids was not significantly altered, whereas in monoglycerides ($P < 0.05$) and diglycerides ($P < 0.01$) it was decreased. For triglycerides, incorporation of [U-^{14}C]PAM was significantly elevated with MEP, although the effect was less in contrast to previous studies done at higher doses (10 mg/kg) (13, 14).

The mechanism underlying increased triglyceride radioactivity was thought to be attributed to an increase in brain palmitoyl-CoA pool, as triglycerides could act as a reservoir for this large acyl-CoA pool once esterification into phospholipids is saturated. However, present results show that 2.5 mg/kg MEP treatment did not affect the size of the palmitoyl-CoA pool ($P > 0.42$) or significantly alter the composition of the various molecular acyl-CoA species (**Fig. 2**). This indicates that acyl-CoA in brain may be tightly regulated and that the size of the pool depends on the equilibrium of acyl-CoA synthesizing and hydrolyzing activities and not carnitine acyltransferase I. This is especially significant because

TABLE 1. Effect of methyl palmoxirate (MEP) given 4 h prior to intravenous infusion of [U-^{14}C] palmitate on distribution of radioactivity into various brain lipids

	% Lipid k*						
Treatment	PL	MG	CHOL	DG	FFA	TG	CE
Vehicle (n = 5)	52.7 ± 5.0	3.1 ± 0.7	7.4 ± 1.0	6.0 ± 0.6	15.1 ± 5.9	11.1 ± 1.2	4.4 ± 1.5
MEP (n = 6)	61.3 ± 0.5	1.6 ± 0.1[a]	6.5 ± 0.2	3.6 ± 0.1[b]	9.5 ± 0.6	15.2 ± 1.3[a]	2.3 ± 0.2

Each value represents mean ± SEM. PL, phospholipids; MG, monoglycerides; CHOL, cholesterol; DG, diglycerides; FFA, free fatty acids; TG, triglycerides; CE, cholesteryl esters.

[a]$P < 0.05$; [b]$P < 0.01$, comparison between MEP-treated and control.

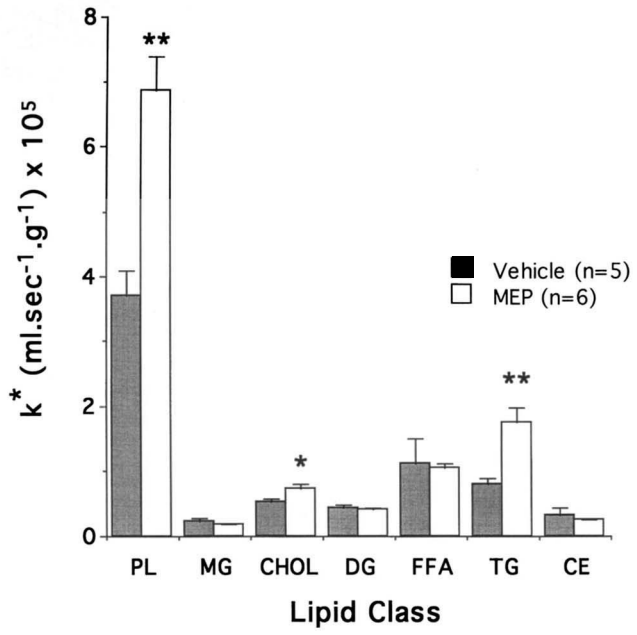

Fig. 3. The effect of MEP (2.5 mg/kg, 4 h pretreatment) on the incorporation coefficient (k*) of [U-^{14}C]palmitate into various lipid classes. Each bar represents the mean ± SEM. Statistical significance ($*P < 0.05$, $**P < 0.01$) was observed between MEP-treated and vehicle.

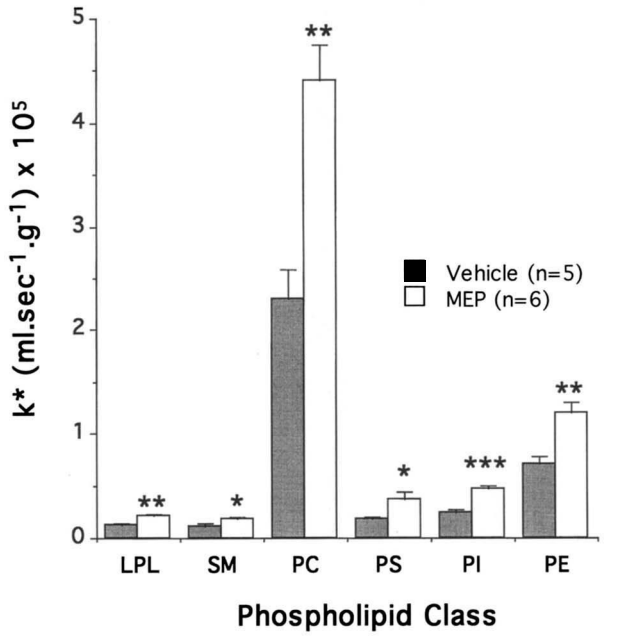

Fig. 4. The effect of MEP (2.5 mg/kg, 4 h pretreatment) on the incorporation coefficient (k*) of [U-^{14}C]palmitate into various phospholipid classes. Each bar represents the mean ± SEM. Statistical significance ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) was observed between MEP-treated and vehicle.

activities of acyl-CoA synthetase and acyl-CoA hydrolase have been shown to be low and extremely high in brain, respectively (21, 22). If the balance of acyl-CoA synthesizing and hydrolyzing enzyme activities are offset, it could potentially alter the acyl-CoA/free CoA ratio and in turn affect incorporation of fatty acids into membrane phospholipids. An elevated acyl-CoA/free CoA ratio is known to inhibit acyl-CoA:lysophospholipid acyltransferase (23, 24). MEP inhibits carnitine acyltransferase I activity, which catalyzes the transfer of fatty acyl residues from CoA to carnitine (20). Because carnitine also serves as a temporary storage (acyl-carnitine) for activated fatty acids (25), triglycerides may act to buffer the acyl-CoA pool when carnitine acyltransferase I activity is inhibited by MEP.

Fatty acid radiotracers have a selective pattern of incorporation into brain phospholipids. Thus radiolabeled PAM is a marker mainly for the *sn*-1 position of phosphatidylcholine (3, 4). The net incorporation coefficient for phospholipids (k^*_{PL}), the product of percent incorporation of [U-^{14}C]PAM into PL and of net k*, was significantly increased by MEP to 85.2% above control levels ($P < 0.01$) therefore raising the signal into this compartment by 1.9 fold (**Fig. 3**). As the phospholipid signal is 4 times greater than the triglyceride signal, radiolabeled PAM with MEP can be used as a marker of brain phospholipid metabolism (and particularly phosphatidylcholine). In addition, MEP treatment significantly increased k* for each phospholipid class, with the greatest changes occurring in phosphatidylserine (102%), phosphatidylinositol (94%), and phosphatidylcholine (90%) **(Fig. 4).** This indicates that the selectivity of [U-^{14}C]PAM to label phosphatidylcholine is maintained with MEP treatment.

MEP is known to inhibit oxidation of fatty acids in liver, heart, kidney, and hemidiaphram (19, 26, 27). Our laboratory has also reported that plasma integrated lipid radioactivity, a reflection of peripheral β-oxidation, was increased significantly in rats that were pretreated with MEP at a dose of 10 mg/kg (13, 14). In the present study, however, the plasma integral in MEP-treated and vehicle-treated rats was 87190 ± 5453 nCi/ml · sec and 103050 ± 8334 nCi/ml · sec ($P > 0.17$), respectively. This suggests that oral administration of MEP at this lower dose should not lead to any abnormal clinical manifestation, such as hepatocellular or muscular lipid accumulation, as reported in carnitine acyltransferase-deficient humans (28–31).

In summary, MEP treatment (2.5 mg/kg, p.o., 4 h pretreatment) can significantly increase the incorporation of [U-^{14}C]PAM into brain lipids, substantially decrease unwanted background signal attributed to radiolabeled aqueous metabolites, and does not change the

concentration of the critical precursor pool. As incorporation of radiolabeled palmitate is related to membrane remodeling and neuroplasticity involving mainly phosphatidylcholine (5–7, 9, 11, 32), its increased distribution into phospholipids with MEP should make it a better marker for imaging structural aspects of brain lipid metabolism in humans by PET.

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