

# No evidence for direct incorporation of esterified palmitic acid from plasma into brain lipids of awake adult rat

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**Abstract** Awake adult rats were given a solution of [9,10-<sup>3</sup>H]palmitate ([<sup>3</sup>H]PAM) by gavage. The appearance of radiolabel in plasma lipid fractions was monitored by thin-layer chromatography at fixed intervals thereafter. At 2 h, the rats were killed by microwave irradiation and radioactivity in whole brain and individual brain phospholipids was determined. In plasma, esterified [<sup>3</sup>H]PAM was mainly associated with triglyceride, phospholipid, and cholesterol ester. Radioactivity appeared to a larger extent in triglyceride than in unesterified fatty acid, suggesting that unesterified [<sup>3</sup>H]PAM in plasma was largely due to release from esterified [<sup>3</sup>H]PAM by lipoprotein lipase hydrolysis. Brain radioactivity could be accounted for entirely by incorporation of unesterified plasma [<sup>3</sup>H]PAM. Esterified [<sup>3</sup>H]PAM in chylomicrons or lipoproteins was calculated to make no measurable contribution using a published value for the incorporation coefficient of [<sup>3</sup>H]PAM into brain in the evaluation. These results suggest that ingested palmitic acid (PAM) in adult rats enters blood as esterified triglyceride within chylomicrons and lipoproteins and, in part, eventually is converted to circulating unesterified PAM. It is the circulating unesterified PAM that is incorporated into brain from blood, whereas esterified PAM within plasma chylomicrons and lipoproteins makes no measurable direct contribution.—**Purdon, D., T. Arai, and S. Rapoport.** No evidence for direct incorporation of esterified palmitic acid from plasma into brain lipids of awake adult rat. *J. Lipid Res.* 1997. **38**: 526–530.

**Supplementary key words** lipoprotein • unesterified fatty acid • phospholipid • triglyceride lipase • incorporation

Unesterified fatty acids circulating in blood, free or bound to albumin, can be taken up and incorporated into brain membrane lipids (1, 2). In particular, we demonstrated significant incorporation of intravenously injected, albumin-bound, unesterified [9,10-<sup>3</sup>H]palmitic acid ([<sup>3</sup>H]PAM) from plasma into the brain of awake rats, principally into choline glycerophospholipids (2, 3). Other potential sources of palmitate for entry into brain phospholipids are plasma acylated palmitate present as lipoproteins, chylomicrons, or lysolecithin (3–5) recycled palmitate released from

brain membrane phospholipids by the action of phospholipases, and palmitate produced by de novo synthesis in brain (3–5). Fatty acids from each source would be expected to enter the brain palmitoyl-CoA pool before being incorporated into brain phospholipids or neutral lipids, or being metabolized by  $\beta$ -oxidation (3, 4).

In plasma, esterified fatty acids are found mainly incorporated in the triglycerides and phospholipids of circulating lipoproteins or chylomicrons (4). Scott and Bazan (6) implicated plasma lipoprotein in the delivery of plasma docosahexaenoate to brain in developing rats. Li, Wetzel, and O'Brien (7) correlated uptake of docosahexaenoate into retina phospholipids with the sequential appearance of radiolabeled docosahexaenoate in chylomicrons and very low density lipoproteins (VLDL) in plasma, after ingestion of the radiolabel by gavage. Receptors for circulating lipoproteins have been shown to reside on cerebrovascular endothelial cells (8, 9), and receptor-mediated uptake of lipoproteins by isolated brain capillary endothelium has been reported (10). Subsequent release of fatty acids in endothelial and neural compartments may occur from intracellular lysosomes (11). Alternatively, lipoprotein lipase on cerebral vascular endothelial cells, acting on lipids of circulating lipoprotein and chylomicrons, may release unesterified fatty acid into the endothelial surface for uptake by cells directly (12, 13). Lipoprotein lipase acting on esterified lipids circulating in plasma or bound to endothelial cells also may release fatty acid into the plasma unesterified fatty acid pool.

Uptake of unesterified plasma fatty acid into brain membrane phospholipid has been quantified in adult

Abbreviations: PAM, palmitic acid; VLDL, very low density lipoproteins; HPLC, high pressure liquid chromatography; UE, unesterified; es, esterified.

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awake rats (3, 14). However, a quantitative assessment of the contribution to brain phospholipid incorporation of fatty acids acylated within circulating lipoproteins or chylomicrons has not been made or compared with the contribution of unacylated plasma fatty acids. Such information would be important for understanding and applying our fatty acid model to brain phospholipid metabolism *in vivo* (4), and for determining, whether circulating lipoproteins and chylomicrons can deliver fatty acids directly to brain in adult animals. We therefore decided to feed [<sup>3</sup>H]PAM to adult male rats to estimate the relative contributions of plasma acylated [<sup>3</sup>H]PAM and unacylated [<sup>3</sup>H]PAM to brain phospholipid radioactivity.

## MATERIALS AND METHODS

### Materials

[<sup>3</sup>H]PAM (16:0 specific activity 47 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Radiochemical purity of [<sup>3</sup>H]PAM was verified as >98% by HPLC (3). Phospholipid and neutral lipid standards were obtained from Avanti (Birmingham, AL) and Deva Biotech (Hatboro, PA).

### Experimental procedures

Experiments were performed according to NIH guidelines on the Care and Use of Laboratory Animals (NIH Publication No. 80-23) and were monitored by the NICHD Animal Care Committee. Male Sprague-Dawley rats (200–300 g) 3 months old were purchased from Charles-River Laboratories (Wilmington, MA) and were maintained *ad libitum* on standard rat chow and water, under a 12-h light/dark cycle (lights on at 0600 h). A rat was anesthetized with sodium pentobarbital (40 mg/kg, *i.p.*), and a polyethylene catheter (PE 50) filled with bicarbonate-buffered physiological saline was tied into the left femoral artery. The wound was closed with application of a local anesthetic, 1% xylocaine. After the trunk of the animal was placed in a loose plaster cast, the cast was positioned on a wooden block and the rat was allowed to recover from anesthesia for 4 h (14).

[<sup>3</sup>H]PAM was dried under nitrogen and resuspended in distilled water containing 3% (w/v) fatty-acid free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) to a final concentration of 2.0 mCi/ml. One-half ml of the [<sup>3</sup>H]PAM solution was administered by gavage followed by 0.15 ml of water. Arterial blood samples were drawn from the left femoral artery at 20-min intervals to determine radioactivity and monitor [<sup>3</sup>H]PAM

in its various forms in plasma. Plasma was collected after each blood sample was centrifuged.

Fifteen sec before the end of an experiment, a final blood sample was taken and the animal was rapidly anesthetized by 33 mg of pentobarbital injected into the femoral artery. The animal was detached from all catheters and immediately killed by focused-beam microwave irradiation (5.5 kW, 3.0 s) (Cober Electronics, Stanford, CT) (14). Within 3 min the brain was removed and homogenized in 20 ml of methanol. Butylated hydroxytoluene (0.01%) (Sigma) was added and the brain was extracted by the method of Folch, Lees, and Sloane Stanley (15). A second extraction of the formic acid-acidified upper phase and protein fractions was performed with chloroform to ensure complete removal of phospholipids and unesterified fatty acids. Blood, plasma, and brain radioactivities were determined by liquid scintillation counting.

Lipids in organic extracts of brain, blood, and plasma were separated by TLC on silica gel plates (Silica Gel 60A TLC plates, Whatman, Clifton, NJ) and were identified by co-chromatography with authentic standards. Neutral lipids were resolved and separated from phospholipids using the solvent system of Skipski et al. (16). Phospholipids were resolved using a solvent system of chloroform–ethanol–triethylamine–isopropanol–water–methanol 30:9:25:25:6:1.5 (v/v) (14). Brain [<sup>3</sup>H] radioactivity for each lipid was corrected for residual blood radioactivity by subtracting the product of cerebral blood volume (ml/g), 2.01 ml/g brain (14), and of whole blood radioactivity.

### Statistics

All values are means ± SEM. Statistical significance ( $P < 0.05$ ) was determined by a two-tailed, unpaired *t*-test.

### Calculations

In order to estimate the relative contributions of plasma unesterified (UE) fatty acid and fatty acid esterified within plasma phospholipids and chylomicrons (ES) to brain phospholipids, we wrote the following equation:

$$dc_{br}^*/dt = k_{UE}^*c_{pl(UE)}^* + k_{ES}^*c_{pl(ES)}^* \quad Eq. 1$$

where  $c_{br}^*$  equals brain concentration of labeled fatty acid,  $c_{pl(UE)}^*$  and  $c_{pl(ES)}^*$  are plasma concentrations of unesterified and esterified radiolabeled fatty acid, respectively,  $k_{UE}^*$  and  $k_{ES}^*$  are the unidirectional incorporation coefficients for unesterified and esterified fatty acid, respectively, and  $t$  is time (3, 4). Integrating equation 1 from the start of fatty acid infusion to time of death  $T$  gives,

$$c_{br}^*(T) = k_{UE}^* \int_0^T c_{pl(UE)}^* dt + k_{ES}^* \int_0^T c_{pl(ES)}^* dt \quad Eq. 2$$

where  $c_{br}^*(T)$  equals brain radioactivity at  $T$ .

Equation 2 can be arranged to provide an expression for  $k_{ES}^*$ ,

$$k_{ES}^* = \frac{c_{br}^*(T) - k_{UE}^* \int_0^T c_{pl(UE)}^* dt}{\int_0^T c_{pl(ES)}^* dt} \quad \text{Eq. 3}$$

It has been calculated from studies in which albumin-bound unesterified [ $^3\text{H}$ ]PAM was injected intravenously in awake rats that the unidirectional incorporation coefficient  $k_{UE}^*$  of unesterified plasma tracer into brain equals  $5.5 \pm 0.3$  (SEM)  $\times 10^{-5}$  ml/(g · sec) (3). We inserted this value for  $k_{UE}^*$  into equation 3 and solved for  $k_{ES}^*$  from experimental measurements of the other terms in the right side of the equation. A value for  $k_{ES}^*$  greater than zero would indicate that esterified fatty acid made a direct contribution to  $c_{br}^*$ . This was not the case (see below).

We also calculated  $k_{UE}^*$  by equation 2 when assuming that  $k_{ES}^*$  equaled zero, and compared the calculated value to that published (3) (see above). It did not differ significantly from the published value (see below).

## RESULTS

### Distribution of [ $^3\text{H}$ ]PAM in plasma lipids

[ $^3\text{H}$ ]PAM in plasma and its distribution among different lipid components over the 2-h period after gavage with [ $^3\text{H}$ ]PAM are shown as average values for a group of six animals (Fig. 1). Total blood radioactivity peaked at 60–80 min after the start of the experiment. Most radiolabeled fatty acid was acylated in plasma within triglyceride, cholesterol ester, and phospholipid. Integrated unesterified [ $^3\text{H}$ ]PAM constituted approximately 15% of the total [ $^3\text{H}$ ]PAM in plasma, the remainder being in the esterified form. The predominant esterified radiolabeled product was triglyceride, which appeared at higher concentrations than the other components, particularly the unesterified fatty acid. These time courses suggest that the unesterified [ $^3\text{H}$ ]PAM was formed mainly by the action of lipases on the [ $^3\text{H}$ ]PAM esterified within circulating chylomicrons or lipoproteins.

### Incorporation of [ $^3\text{H}$ ]PAM into brain phospholipid

We previously determined the unidirectional incorporation coefficient  $k_{UE}^*$  (Eq. 1) for unesterified [ $^3\text{H}$ ]PAM into brain lipid from plasma during intravenous infusion of unesterified [ $^3\text{H}$ ]PAM (3). In that experiment, all [ $^3\text{H}$ ]PAM was in the unesterified form in

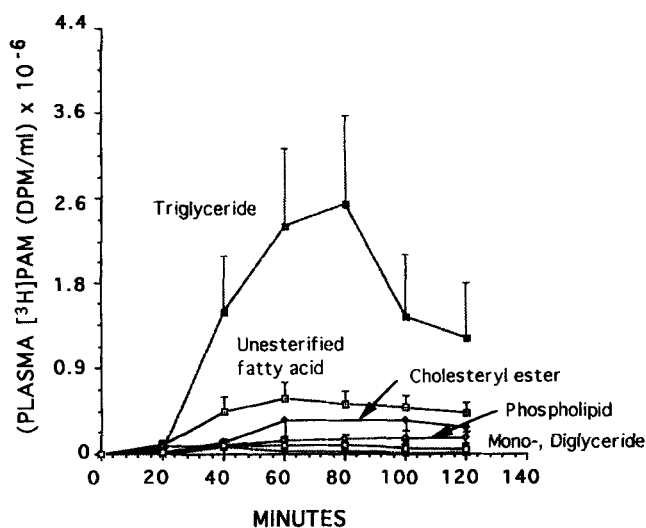


Fig. 1. [ $^3\text{H}$ ]PAM in different fractions of plasma following oral administration of 1.0 mCi [ $^3\text{H}$ ]PAM in albumin. The distribution of radioactivity in different lipid components is shown for group means  $\pm$  SEM ( $n = 6$ ) where the peak ordinate value is  $4.4 \times 10^6$  dpm/ml plasma.

plasma and thus  $k_{ES}^*$  could not be examined. A value of  $5.5 \pm 0.3 \times 10^{-5}$  ml/(g · sec) (mean  $\pm$  SEM,  $n = 12$ ) was reported (3). Calculating  $k_{UE}^*$  from equation 2 when assuming that  $k_{ES}^*$  equaled zero gave an estimate of  $4.6 \pm 1.7 \times 10^{-5}$  ml/(g · sec), a value not significantly different from the value reported by Grange et al. (3). Thus, all brain radioactivity could be explained as coming from unesterified [ $^3\text{H}$ ]PAM in plasma when letting  $k_{UE}^* = 0$ .

Inserting the value for  $k_{UE}^*$  into equation 3, together with values for the integral of unesterified [ $^3\text{H}$ ]PAM in plasma ( $c_{pl(UE)}^*$ ) (Fig. 1), gave a mean value for brain phospholipid radioactivity not significantly different from our observed value ( $14.8 \pm 3.4 \times 10^4$  dpm, mean  $\pm$  SEM). Thus the estimate for  $k_{ES}^*$  was not significantly different from zero.

In Table 1 are summarized unidirectional incorporation coefficients for unesterified [ $^3\text{H}$ ]PAM in total brain and individual brain phospholipids. Most radiotracer

TABLE 1. Incorporation coefficients ( $k_{UE}^*$ ) from plasma unesterified [ $^3\text{H}$ ]PAM into brain phospholipid

Brain Compartment	$k_{UE}^*$ $\text{ml}/(\text{g} \cdot \text{sec}) \times 10^5$
Total phospholipid	$4.6 \pm 1.7$
Choline phospholipids	$3.6 \pm 1.2$
Phosphatidylserine	$0.1 \pm 0.05$
Phosphatidylinositol	$0.2 \pm 0.1$
Ethanolamine phospholipids	$0.7 \pm 0.3$

Values are means  $\pm$  SEM for  $n = 6$ . Only integrated plasma radioactivity of unesterified [ $^3\text{H}$ ]PAM was used in the calculation.

was incorporated into brain phosphatidylcholine compared with other phospholipids. These results agree with previous findings obtained in awake adult rats using intravenous infusion of unesterified [ $^3\text{H}$ ]PAM (3).

## DISCUSSION

Our aim was to compare incorporation of esterified and unesterified [ $^3\text{H}$ ]PAM from plasma into brain. Although esterified and unesterified forms of [ $^3\text{H}$ ]PAM are found in plasma following ingestion of [ $^3\text{H}$ ]PAM in adult awake rats, incorporation of [ $^3\text{H}$ ]PAM into brain can be explained entirely by uptake of the unesterified radiolabel. We calculated a unidirectional incorporation coefficient from the unesterified [ $^3\text{H}$ ]PAM radioactivity found in plasma (Fig. 1) and found that its value was not significantly different from the  $k_{\text{UE}}^*$  calculated by Grange et al. (3), under conditions where  $k_{\text{ES}}^*$  was taken as zero. In the work of Grange et al. (3), the presence of only unesterified [ $^3\text{H}$ ]PAM in plasma was ensured by infusion of unesterified radiolabel and the short time course of the experiment. In the present study, unesterified [ $^3\text{H}$ ]PAM resulted from the action of enzymes acting on the esterified fatty acid fraction in plasma, principally composed of radiolabeled triglyceride. The lack of significant difference between  $k_{\text{UE}}^*$  values in this work and in the study of Grange et al. (3) emphasizes the utility of the unidirectional incorporation coefficient for describing fatty acid uptake and incorporation into membrane phospholipids. The unidirectional incorporation constant  $k_{\text{UE}}^*$  can be used to calculate brain radioactivity according to equation 2 when  $k_{\text{ES}}^* = 0$ . Calculated brain radioactivity is not significantly different from the actual brain radioactivity, again emphasizing the unesterified plasma fatty acid fraction as the source of radiolabel for brain membrane phospholipid.

Solving for  $k_{\text{ES}}^*$  using equation 3 also indicated that no contribution was made by the esterified component in plasma. This means that  $k_{\text{ES}}^* \ll k_{\text{UE}}^*$  in equation 3. Li et al. (7), using a similar approach for docosahexaenoate, showed that chylomicron-associated radioactivity peaked at 2 h after administration of tracer by gavage, while radiolabeled VLDL was only starting to appear. As our experiments lasted only 2 h, it remains possible that a combination of chylomicrons and VLDL would contribute after 2 h to direct transfer of esterified [ $^3\text{H}$ ]PAM to brain. Palmitate in plasma 1-palmitoyl-2-lyso PtdCho may also contribute to the brain palmitoyl-CoA pool (5).

In the present study, a fraction of total plasma [ $^3\text{H}$ ]PAM was unesterified (Fig. 1). It is generally as-

sumed that when intestinal unesterified fatty acids are processed by enterocytes, they are covalently incorporated, mainly into the triglyceride of chylomicrons and of intestinal lipoprotein, and are secreted into the lymph (17, 18), although a small unesterified component may be released into the portal vein (19). Most of the unesterified [ $^3\text{H}$ ]PAM in plasma probably was released from esterified sources by lipases. Some workers have demonstrated that fatty acids released from endothelial-bound chylomicrons or VLDL by lipoprotein lipase can enter the circulation if the fatty acids exceed cellular requirements (20). Fatty acids also can induce the release of endothelial-bound lipoprotein lipase into the circulation, where the enzyme could act on substrate to further release fatty acid (20, 21). Indeed, when palmitoyl-radiolabeled chylomicrons (22, 23) and VLDL (24) are infused intravenously in rats, free radiolabeled palmitate is found in plasma. Therefore, unesterified fatty acids released from covalent linkages in the endothelial-bound and fluid phases can explain the presence of unesterified [ $^3\text{H}$ ]PAM found in plasma that we found in this work.

In summary, ingested [ $^3\text{H}$ ]PAM appears initially in intestinal lymph incorporated in triglycerides (Fig. 1) (17, 18) of chylomicrons and in intestinal VLDL. Subsequently, action of lipase on this esterified [ $^3\text{H}$ ]PAM releases unesterified [ $^3\text{H}$ ]PAM into the plasma, from where it can be incorporated into brain phospholipid. Unesterified [ $^3\text{H}$ ]PAM is the only measureable plasma source of incorporated brain [ $^3\text{H}$ ]PAM. The palmitate contributed from plasma to the brain palmitoyl-CoA pool represents a small part of total brain palmitate flux into phospholipid (3). ■■

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