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Inter- and intra-fat pad variation in vascularization and the release of ^{14}C-labelled fatty acids in mice

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Adult laca mice were dosed orally with 150 μl whole milk containing 2·5 μCi of either labelled stearic acid ([1-^{14}C]18:0; *n* 20) or labelled linoleic acid ([1-^{14}C]18:2; *n* 20). The mice were killed in groups of four at 6, 12, 24, 48 and 96 h following dosing and samples of perirenal and epididymal fat pads were taken from both inner and outer sites at each location. Significant differences in the rate of loss of label between sites were found. No differences ($P = 0·018$) between fat pad locations (epididymal *v.* perirenal) were found. A significant interaction between rate of loss of labelled fatty acid and site ($P = 0·019$) reflected the fact that between-site variations in this context were confined to labelled linoleic acid. In a second study weanling adult (*n* 10) and adult (*n* 10) mice were killed and their epididymal and perirenal fat pads prepared for histological examination. Both transverse and longitudinal sections were taken at the inner and outer sites of each fat pad location. Following staining, both the size and number of blood vessels were measured using computer-linked microscopy. In all instances there were significant differences between sites with the inner site consistently showing greater numbers and areas of blood vessels. In general the number of blood vessels in the inner site tended to be greater in older mice, while the reverse was seen for younger mice. The results lend support to the concept of multiple pools of triacylglycerol-fatty acids in adipose tissue such that the main determinant of short-term supply of essential fatty acids is the quantity recently ingested.

Adipose tissue turnover: Fat pad vascularization: Mice

Almost all nutrients are partitioned within the body into different pools which exist in some form of equilibrium. In the case of triacylglycerol (TAG) the main pools are the plasma lipoproteins and the adipose tissue. TAG enters the adipose tissue pool from plasma chylomicrons and very-low-density lipoproteins (VLDL) but leaves in the form of non-esterified fatty acids (NEFA) which are used for energy or for hepatic resynthesis of VLDL. The total daily dietary input of TAG in man is about 100 g/d which has to equilibrate with an adipose tissue pool of about 10 kg. Previous studies have indicated that in the postprandial phase the fatty acid composition of the NEFA reflects that of the ingested TAG (Heimberg *et al.* 1974). This would indicate that ingested lipid does not equilibrate with a large single homogeneous adipose tissue TAG pool but that the ingested lipid enters a specific adipose tissue TAG pool for subsequent withdrawal as NEFA in the later postprandial phase. This concept of two or more adipose tissue TAG pools has been extensively studied (Hirsch *et al.* 1960; Kerpel *et al.* 1961; Stein & Stein, 1962; Angel, 1970; Winand *et al.* 1971; Zinder *et al.* 1973). The nutritional significance of this concept is considerable in that the main determinant of short-term supply of essential fatty acids is the quantity recently ingested. Adipose tissue biopsies may well reveal data on the long-term habitual intake of essential fatty acids (Wood *et al.* 1984; Kearney *et al.* 1989) but cannot be used to infer daily availability, if two pools of adipose TAG do exist.

The nature of the proposed two-pool structure of adipose tissue TAG is unclear. Several

authors have suggested that different adipose tissue sites contribute to this compartmentalization of adipose tissue TAG on the basis of variation in cell size, blood flow and degree of innervation (Hamosh *et al.* 1963; Munkner, 1963; Gellhorn & Benjamin, 1965; Hartman & Christ, 1978; Smith, 1985). To date there is little information available on intra-adipose tissue site variation (Hollenberg & Vost, 1968; Hill & Baker, 1983; Baker *et al.* 1984) and it remains possible that intra-adipose tissue variation could be as significant as inter-adipose tissue TAG compartmentalization. Thus, the present study set out to examine both intra- and inter-fat TAG and to examine any potential relationship with the degree of vascularization.

MATERIALS AND METHODS

Animals and their management

Weanling (3 weeks old) and adult (3 months old) male laca mice were used (Bioresources Unit, Trinity College, Dublin, Republic of Ireland). The mice were housed in groups of four in standard mouse boxes with *ad lib.* access to fresh food and water daily. In Expt 1 the high-fat diets contained (g/kg): casein 216, DL-methionine 3, butter 302, maize oil 26, starch 184, sucrose 162, cellulose 5, mineral mix 42, vitamin mix 12. The mineral and vitamin mixes were designed to meet the established nutrient requirements of laboratory mice (American Institute of Nutrition, 1977). In Expt 2 a standard laboratory mouse diet was used. All animals were killed by cervical dislocation under light halothane anaesthesia.

Preparation of labels for oral dosing

Portions (50 μCi) of [1-^{14}C]linoleic acid (18:2) and [1-^{14}C]stearic acid (18:0), obtained from Amersham International (UK), were removed from cold storage at $-20°$, the solvent evaporated under N_2, the lipid removed from the phial in four washes with chloroform into a test-tube, dried again under N_2 and reconstituted in 250 μl warm ethanol and 4·75 ml fresh cow's milk. The mixture was vortexed and stored at $-4°$ for no more than 8 h before use.

Expt 1. Uptake and release of 1-^{14}C-labelled fatty acids

Forty adult (3 months old) male laca mice were put on a high-fat diet as previously described, for 2 weeks before oral dosing. Half the mice were dosed orally with [1-^{14}C]18:0 while the remainder were given [1-^{14}C]18:2. Each mouse was given 150 μl of the labelled solution containing 2·5 μCi 1-^{14}C-labelled fatty acid. The mice were killed in groups of four at 6, 12, 24, 48 and 96 h post-dosing. Perirenal and epididymal fat pads were excised, weighed and immediately frozen. From the frozen pad, the following samples were taken. A small sample was taken from the longitudinal extremity (the tip); the pad was then cut along a central transverse line into two halves and a thin slice cut along the length of the exposed face of the transverse section. A sample was taken from the edge of the thin slice (the outer) and one from the very centre (inner). These samples (the tip, the outer and the inner) were weighed and extracted twice for 3 h each in chloroform–methanol (2:1, v/v). The extracted lipid was dried under N_2. Scintillation fluid (10 ml; hydrofluor; National Diagnostics) was added and left overnight in the dark at 4° before counting in a Packard 1500 Tricarb liquid-scintillation analyser with automatic quench compensation. Throughout the present paper the term used to define different fat pads is 'fat pad location' while different samples from within a given location are defined as 'fat pad site'.

Expt 2. Measurement of the degree of fat pad vascularization

Twenty male laca mice, ten weanlings (3 weeks old) and ten adults (3 months old) were used. The mice were killed by cervical dislocation and both pairs of epididymal and perirenal fat pads were removed, weighed, fixed in formaldehyde (100 ml/l) for 24 h,

dehydrated in ascending grades of alcohol (700, 900, 1000 ml/l) and cleared in chloroform for 1–2 h. The pads were impregnated in paraffin wax (56–58°). Sections, each 5 μm, were taken using a microtome 820 (Spencer) from outer and inner sites of the pads. Both transverse and longitudinal sections were taken at each site. They were then rehydrated with descending alcohol series and stained with haematoxylin under standard conditions.

Analysis of the slides was made using a Kontron Image Analyser (MOP Videoplanner) linked to a Nikkon (Labophot) microscope. Measurements of blood vessel numbers and areas were taken for twelve defined microscopic fields in both inner and outer sites. The mean of twelve readings constituted one value for each mouse. Histologically adequate sections were not obtained for all mice. In the case of transverse sections data were available for seven young and six old mice and in the case of longitudinal sections for nine mice in each age category.

Statistical analysis

In Expt 1 the results in hundreds of disintegrations/min (10^2 dpm) were converted to the square root to stabilize the variance. The fact that the standard deviations of these values tended to decrease with time indicated that this stabilization was not fully achieved. The data for Expt 1 were analysed by a repeated measures analysis of variance using the SAS statistical package program (SAS Institute Inc., 1985). Where appropriate the P values were adjusted using the Greenhouse–Geisser procedure (Greenhouse & Geisser, 1959). In Expt 2 the numbers and areas of blood vessels were not normally distributed and, thus, the $\log_{10}$ of each value was used. In addition 0·5 μm was added to each value before log conversion to take account of zero values. The data were then analysed for both longitudinal and transverse sections by a three-way analysis of variance with fat pad location (epididymal and perirenal; L), fat pad site (inner, outer, tip; S) and age (A) as the three main factors.

RESULTS

The mean body weight of mice in Expt 1 increased from 29·3 (SD 4·9) g at the start of the high-fat diet to 34·1 (SD 3·7) g after 2 weeks feeding. The rates of loss of labelled fatty acids from different sites on epididymal and perirenal fat pad locations are given in Table 1 and the analysis of variance given in Table 2. The effect of S was significant ($P < 0·008$), as was that of time (T; $P < 0·001$). No significant effect of S or labelled fatty acid source (F) was observed. Only one interaction term was significant, that of S × F × T ($P = 0·006$). These effects can be best demonstrated in Fig. 1 where the data for pad location (not significantly different) have been pooled. Whereas in the case of [1-^{14}C]18:0 the time-related loss of label was similar for each fat pad site (Fig. 1(a)), clear differences were evident between sites in the rate of loss of [1-^{14}C]18:2, the inner site showing the most rapid rate of loss of label.

Table 3 shows the numbers and areas of blood vessels in transverse and longitudinal sections of outer and inner sites of epididymal and perirenal fat pads. Results of the analysis of variance are given in Table 4. In all instances there were significant differences between sites, with the inner site consistently showing greater numbers and areas of blood vessels ($P < 0·001$–$P = 0·002$). For numbers of blood vessels a significant interaction between the effects of A and S was found for both the transverse and longitudinal sections ($P = 0·021$ and $P = 0·027$ respectively). A similar interaction was found for blood vessel areas in the transverse section ($P = 0·002$). Thus, younger mice had greater numbers of blood vessels than older mice in the outer sites in both sections (1·08 *v.* 0·70) but had fewer numbers of blood vessels in the inner sites (1·34 *v.* 1·62). In the longitudinal section the older mice had greater blood vessel areas in the outer site (6·6 *v.* 5·6) but smaller blood vessel areas in the inner site (7·2 *v.* 8·3). The significant interaction between L and S observed for blood vessel

Table 1. *Loss of [1-^{14}C]stearic acid (18:0) and [1-^{14}C]linoleic acid (18:2) from the tip, inner and outer sites of epididymal and perirenal fat pads at 6, 12, 24, 48 and 96 h after labelled fatty acid intubation**

(Values are means for four rats)

Fat pad location...	Epididymal $(10^2 \text{ dpm})^{0.5}$						Perirenal $(10^2 \text{ dpm})^{0.5}$					
Fat pad site...	Tip		Inner		Outer		Tip		Inner		Outer	
1-^{14}C-labelled fatty acid...	18:0	18:2	18:0	18:2	18:0	18:2	18:0	18:2	18:0	18:2	18:0	18:2
Time-period post-intubation (h)†												
6	67	58	40	53	65	53	61	42	52	64	51	59
12	49	45	52	40	49	40	49	47	44	37	46	44
24	33	34	39	28	36	28	37	45	32	31	35	40
48	27	34	24	27	30	27	25	34	25	23	29	29
96	21	25	19	24	22	24	25	31	23	17	26	33

dpm, disintegrations/min.
* For details of procedures, see p. 738.
† Statistical analysis is presented in Table 2

Table 2. *Analysis of variance of the effects of fat pad location (perirenal and epididymal; L), fat pad site (tip, inner and outer; S) on the time-related release of [1-^{14}C]stearic acid (18:0) and [1-^{14}C]linoleic acid (18:2) from adipose tissue in adult mice**

Source of variation	df	Mean square	Statistical significance of effect: *P*
Fatty acid (F)	1	49·2	0·704
Time (T)	4	8079·6	0·001
F × T	4	200·5	0·666
Error	30	596·6	—
L	1	67·9	0·474
L × F	1	233·1	0·189
L × T	4	78·4	0·661
L × F × T	4	65·2	0·744
Error	30	129·3	—
S	2	596·6	0·008
S × F	2	16·7	0·833
S × T	8	18·8	0·990
S × F × T	8	345·7	0·006
Error	60	107·6	—
L × S	2	11·5	0·874
L × S × F	2	135·5	0·258
L × S × T	8	130·1	0·251
L × S × F × T	8	68·9	0·675
Error	60	97·7	—

* For details of procedures, see pp. 738–739.

numbers in the longitudinal section ($P = 0{\cdot}039$) is reflected in the fact that the number of blood vessels in the outer site of the epididymal fat pad was lower than the numbers in the outer site of the perirenal fat pad (0·88 *v.* 1·01) while the reverse was seen for the inner site (1·56 *v.* 1·38).

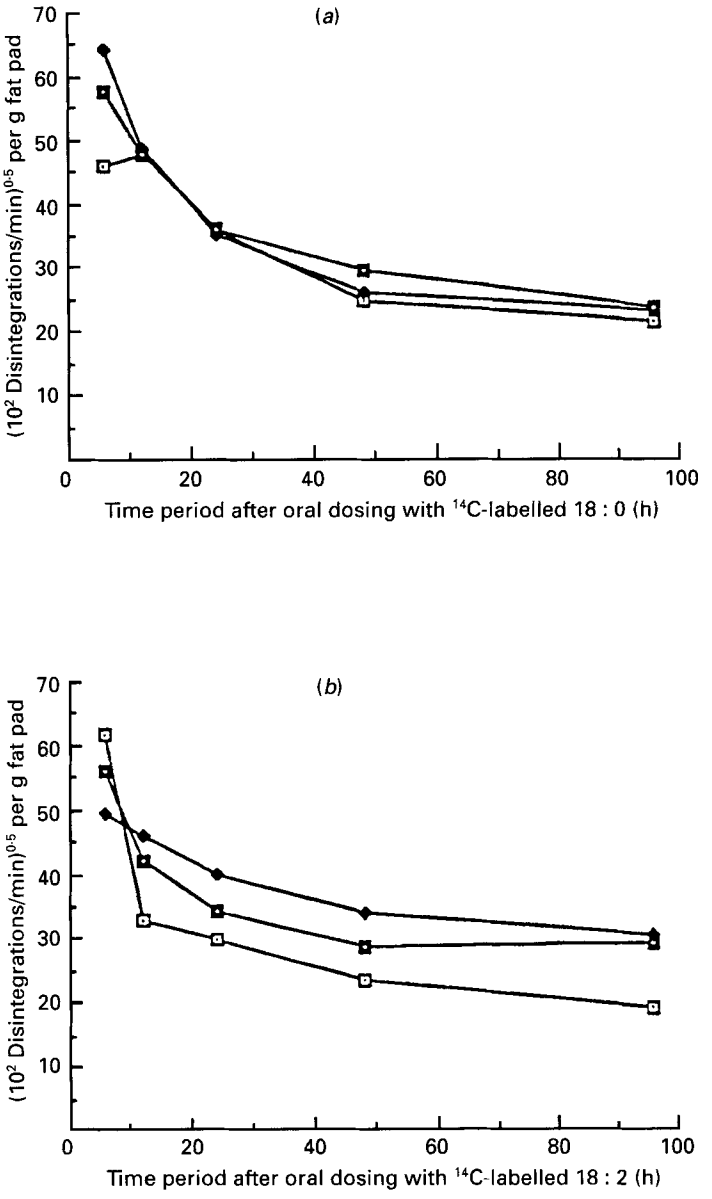

Fig. 1. Rate of loss of (a) ^{14}C-labelled 18:0 and (b) ^{14}C-labelled 18:2 from the inner (—□—), tip (—◆—) and outer (—■—) sites of adipose tissue fat pads (pooled data from epididymal and perirenal fat pads).

DISCUSSION

The present study was designed to examine the possibility that metabolic activity might vary between and within fat pads, that such variation might relate to vascularization and that these findings might reveal something of the nature of different pools of TAG in adipose tissue. The degree of vascularization of the inner sites of fat pads was found to be considerably greater than that of the outer site. This applied to the area of blood vessels and the numbers of blood vessels. Where variation occurred between sites with respect to rates of label loss the rate of loss was greatest from the inner site. This would partly support

Table 3. *No. of blood vessels* ($log_{10}(n+0{\cdot}5)$) *and area of blood vessels* ($log_{10}(\mu m^2+0{\cdot}5)$) *found at outer and inner sites of transverse and longitudinal sections of epididymal and perirenal fat pads in young and old mice**†

(Mean values and standard deviations)

	Transverse								Longitudinal							
	Area				No.				Area				No.			
	Young		Old		Young		Old		Young		Old		Young		Old	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Epididymal																
Outer	5·9	0·8	5·4	2·5	1·26	0·29	0·66	0·75	5·5	1·3	6·7	1·0	0·86	0·63	0·70	0·34
Inner	7·3	0·9	7·9	1·1	1·41	0·50	1·84	0·39	8·5	0·5	6·8	0·9	1·42	0·47	1·69	0·42
Perirenal																
Outer	5·1	1·2	5·1	2·0	1·02	0·35	0·58	0·74	5·7	2·2	6·5	0·8	1·18	0·48	0·84	0·41
Inner	7·6	0·9	7·6	1·0	1·33	0·71	1·38	1·38	8·1	0·7	7·6	1·0	1·21	0·42	1·54	0·39

* For details of procedures, see pp. 738–739.
† Statistical analysis is presented in Table 4.

the hypothesis that the greater the degree of vascularization the greater the rate of metabolic activity, that is the rate of uptake of labelled fatty acids is greatest in such regions and the rate of loss is most rapid. However, between-site variation in the rate of label loss was seen only with 18:2.

In attempting to explain this difference between the rate of loss of these two fatty acids several factors need to be considered. The first is that the key enzyme responsible for hydrolysis of TAG in adipose tissue, hormone-sensitive lipase (*EC* 3.1.1.3), acts preferentially on the *sn*-1 and *sn* − 2 positions of TAG, yielding 1, 2 and 2,3-diacylglycerol and 2-monoacylglycerol (Belfrage, 1985). The second is that in most mammalian (excluding the pig) adipose tissue TAG, 18:2 is contained predominantly on the *sn*-2 position while 18:0 is found predominantly on the *sn*-1 position (Small, 1991). It is conceivable, therefore, that in regions of adipose tissue where vascularization tends to be low, such as the tip or outer sites, the activity of hormone-sensitive lipase is lower. This may not be of much significance for fatty acids such as 18:0 located preferentially in the *sn*-1 position but it may delay the release of 18:2 contained in the *sn*-2 position. Re-esterification of liberated NEFA back to TAG is substantial under normal conditions and is considerable even when adipose tissue is stimulated by catecholamine, nerve stimulation or exercise (Vernon & Clegg, 1985). Several authors have shown that the release of NEFA from adipose tissue is reduced when blood flow through the tissue is restricted leading to an increase in re-esterification (Kovach *et al.* 1976; Belfrage *et al.* 1979; Burlow & Madsen, 1981). It remains possible, therefore, that the differential in the rates of loss of 18:0 and 18:2 from different sites of the two adipose tissue locations may relate to differences in their positional distribution on adipose tissue TAG. This is but one possibility and others may be equally worthy of consideration.

For both fatty acids the rate of loss of label from adipose tissue showed a clear biphasic response. This biphasic response would tend to support the theory that in adipose tissue two pools of TAG are found, one more labile than the other. This could, however, be confounded by the possibility that label lost from the fast-turning pool was recycled largely into the slower-turning pool, thus underestimating the metabolic activity of the latter. However, the findings of Klein *et al.* (1980) would favour the interpretation of this biphasic

Table 4. *The analysis of variance of fat pad location (L), fat pad site (S) and age (A) on the numbers ($log_{10}(n+0{\cdot}5)$) and areas (μm^2) of blood vessels in transverse and longitudinal sections of mouse epididymal and perirenal fat pad locations**

	Transverse section						Longitudinal section					
	Area of blood vessels			No. of blood vessels			Area of blood vessels			No. of blood vessels		
Source of variation	df	Mean square	Statistical significance of effect: *P*	df	Mean square	Statistical significance of effect: *P*	df	Mean square	Statistical significance of effect: *P*	df	Mean square	Statistical significance of effect: *P*
A	1	0·226	0·860	1	0·258	0·547	1	0·019	0·935	1	0·016	0·828
Error	11	6·917	—	11	0·669	—	16	2·695	—	16	0·329	—
L	1	0·166	0·719	1	0·605	0·029	1	0·383	0·560	1	0·012	0·781
A L interaction	1	0·601	0·496	1	0·043	0·518	1	0·663	0·445	1	0·017	0·740
Error	11	1·212	—	11	0·096	—	16	1·039	—	16	0·148	—
S	1	54·05	0·002	1	4·830	0·001	1	44·419	0·000	1	5·389	0·000
A S interaction	1	3·27	0·347	1	1·873	0·021	1	18·180	0·002	1	1·343	0·027
Error	11	3·39	—	11	—	—	16	1·262	—	16	0·225	—
L S interaction	1	0·057	0·842	1	0·042	0·656	1	0·163	0·566	1	0·738	0·039
A L S interaction	1	0·246	0·681	1	0·224	—	1	1·014	0·164	1	0·067	0·507
Error	11	1·377	—	11	0·200	—	16	0·474	—	16	0·146	—

* For details of procedures, see pp. 738–739.

response as being truly indicative of two pools of adipose tissue TAG. These authors interesterified maize oil containing the inert fatty acid marker 13-methyltetradecanoic acid (13-MTD). This fatty acid is not subject to oxidation, elongation or desaturation and is rapidly incorporated into rat adipose tissue TAG with a half-life of incorporation of 4·5–6·5 d. The rate of loss of 13-MTD showed a biphasic response for all adipose tissue sites (subcutaneous, postabdominal, mesenteric, perirenal, epididymal and pericardiac) and the half-life of loss of 13-MTD ranged from 9 to 18 d, averaging 14 d. Thus, the average rate of label loss was considerably slower than the average rate of label gain (half-lives of 14 and 5 d respectively). The pattern of adipose tissue uptake of 13-MTD in the rat study of Klein *et al.* (1980) and that of the uptake of 18:2 in the human study of Dayton *et al.* (1966) are quite similar. Both show a steady rise reaching an upper asymptote slightly below the level in the diet. Taken together, these findings and the results of the present study would indicate that dietary fatty acids are incorporated into adipose tissue TAG at a steady rate but their rate of loss is much slower and is clearly biphasic. Blanchette-Mackie & Scow (1981) have presented evidence that channels exist at the site of capillary–adipocyte contact through which NEFA must be transferred either entering or leaving the adipocyte. It remains possible that such proposed channels could be the main area of activity of lipoprotein lipase (*EC* 3.1.1.34) which liberates NEFA from chylomicrons and VLDL; thus, the entry of NEFA is facilitated. On entry into the adipocyte the NEFA are converted into TAG in the peripheral cytoplasmic region of the adipocyte. Newly synthesized TAG must now diffuse through the lipid droplet. If that rate of diffusion is slow, then, in the short term, newly synthesized TAG would be susceptible to lipolysis back to NEFA. Ookthens *et al.* (1987) have studied the short-term movement of NEFA within the different lipid pools in adipose tissue. They observed a large, slowly turning pool which is not labelled in the short term (60 min) in studies using radiolabelled palmitate injected into rat adipose tissue extracellular space. The labelled fatty acid was rapidly taken up into a small TAG fraction which underwent rapid recycling back to free fatty acids. In the short term, therefore, NEFA entering the adipocyte are likely to enter the TAG pool most susceptible to lipolysis. In the longer term, as the newly synthesized TAG pool slowly equilibrates with the bulk of the lipid droplet, its accessibility to the lipolytic process declines. Thus, while uptake into the adipocyte is orderly and relatively rapid, the longer-term exit is more random than ordered and, therefore, is bound to be slower.

The results of the present study, thus, lend some support to the hypothesis that adipose tissue TAG does not exist in one single homogeneous pool. They also show a much greater level of vascularization in the inner sites of perirenal and epididymal fat pads. Notwithstanding these considerable differences, the rates of loss of the labelled fatty acids were not greatly dissimilar between sites, even where statistically significant differences were found. It is highly probable, therefore, that other factors such as adipocyte size and degree of innervation play an equal or greater role in determining within-fat-pad variability in metabolic activity.

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