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Effects of lactation and removal of pups on the rate of triacylglycerol/fatty acid substrate cycling in white adipose tissue of the rat

Per HANSSON,*†‡ Eric A. NEWSHOLME† and Dermit H. WILLIAMSON*
*Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, and †Department of Biochemistry, South Parks Road, Oxford OX1 3QU, U.K.

The rate of the triacylglycerol/fatty acid substrate cycle was measured *in vivo* in adipose tissue of virgin and lactating rats with pups removed. The rate decreased by 70% in adipose tissue of lactating rats and increased 9-fold on removal of the pups. Similar differences in cycling rate were seen in adipose tissue incubated *in vitro* in the presence of isoprenaline.

INTRODUCTION

Lactation and its termination (weaning) constitute two situations where major changes in adipose-tissue metabolism occur within a short time (for reviews, see Williamson, 1980; Vernon & Flint, 1983). The alterations in adipose-tissue metabolism during lactation include decreased lipogenesis (Agius *et al.*, 1979; Flint *et al.*, 1981), decreased activity of lipoprotein lipase (Hamosh *et al.*, 1970; Flint *et al.*, 1981) and decreased removal of dietary fat (do Nascimento & Williamson, 1986). The overall effect of these changes in lactation is to conserve energy for milk-fat synthesis in the mammary gland, and they result in a decrease in adipose-tissue mass (Spray, 1950; Steingrimsdottir *et al.*, 1980; Naismith *et al.*, 1982; Moore & Brasel, 1984), despite the hyperphagia which accompanies lactation (Fell *et al.*, 1963). Termination of lactation is associated with a reversal of these metabolic changes and rapid repletion of adipose-tissue stores (Vernon & Finley, 1986).

The adipose-tissue mass is dependent on the difference between the rate of esterification of fatty acids (derived from exogeneous sources or synthesized within the tissue) and the rate of lipolysis. It has been known for some time that the two processes can occur simultaneously, that is, a significant proportion of the fatty acids released are immediately re-esterified (Steinberg, 1963). This process of lipolysis and re-esterification is known as the triacylglycerol/fatty acid substrate cycle. One postulated role of substrate cycling is to increase the sensitivity of the control mechanisms, and the triacylglycerol/fatty acid cycle in adipose tissue may increase the sensitivity of the mechanism that controls the rate of deposition of triacylglycerol, for example during the absorption period after a meal (Brooks *et al.*, 1982, 1983; Newsholme & Leech, 1983). Furthermore, it has been demonstrated that the higher the rate of substrate cycling, the greater is the improvement in sensitivity (Newsholme & Crabtree, 1976; Crabtree & Newsholme, 1985).

Since adipose tissue may be metabolically less active during lactation, the rate of triacylglycerol/fatty acid cycling in this tissue *in vivo* has been measured during and immediately after lactation. For comparison, the rate of cycling has been measured in normal rats during starvation. In addition, the effects of a β-adrenoreceptor agonist on the cycling rate have been measured in adipose tissue removed from lactating rats and after removal of pups.

EXPERIMENTAL

Female Wistar rats were fed on a diet (Special Diet Services, Witham, Essex, U.K.) of approximate composition (by wt.) 52% carbohydrate, 21% protein and 4% fat; the residue was non-digestible material. They were kept at 20–22 °C on a 07:30–19:30 h light cycle with free access to chow and water unless otherwise stated. Lactating rats (body wt. 310 ± 25.5 g) were kept alone with their pups and were studied 11–15 days *post partum*. Removal of pups (maternal body wt. 345 ± 41.3 g) was also performed after 11–15 days of lactation, and experiments were carried out 2 days later. Virgin control rats (body wt. 209 ± 9.5 g) were not caged singly.

All experiments were carried out between 09:00 and 11:30 h. Each animal was injected with 6 mCi of ^3H_2O (0.6 ml) intraperitoneally, and after 55 min the rat was anaesthetized with pentobarbital (60 mg/kg body wt.) intraperitoneally. At 60 min, blood was taken by aortic puncture and the parametrial white adipose tissue was excised and freeze-clamped (Wollenberger *et al.*, 1960). Some rats received a tail-vein injection of Tyloxapol [Triton WR 1339; 1 ml of a 10% (w/v) aqueous solution], which decreases the rate of removal of triacylglycerol from the plasma via lipoprotein lipase activity (Scanu, 1965). This was done under light ether anaesthesia 1 h before administration of ^3H_2O. Control rats received 1 ml of 0.9% NaCl. Plasma triacylglycerol concentrations were determined as glycerol (Eggstein & Kreutz, 1966) after saponification.

The frozen tissue (about 400 mg, in duplicate) was dropped into 15 ml of chloroform/methanol (2:1, v/v) and left overnight, after which it was gently squashed and insoluble material removed. The lipid extract was then

* To whom requests for reprints and correspondence should be sent, at present address: Department of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden.

washed three times, first with 3.75 ml of water, then twice with 3.75 ml of methanol/water (1:1, v/v). Separate experiments confirmed that three washes remove all accompanying ^3H_2O (cf. Brooks, 1981). The remaining lipid extract was dried under N_2, then re-solubilized in hexane/diethyl ether (4:1, v/v) and applied to mini-columns of Florisil (100–200 mesh; B.D.H.). Each sample was eluted with 20 ml of the same solvent and was again dried under N_2. Although the introduction of Florisil chromatography increases the time taken to complete the overall procedure, it decreased the quenching of radioactivity during measurements by liquid-scintillation counting. In addition, polar lipids are removed by the Florisil, although preliminary experiments established that there was no detectable incorporation of ^3H into such lipids. The small amount of cholesterol ester in white adipose tissue is co-eluted with triacylglycerol, but preliminary experiments demonstrated that this contributes a negligible amount (< 5%) of radioactivity.

To each sample were added 3 ml of ethanol and 3 ml of 30% (w/v) KOH, together with a known tracer amount (about 600 d.p.m.) of [^{14}C]glycerol. Samples were saponified at 70 °C for 2 h. After cooling, they were acidified with 1.8 ml of 60% (w/v) $HClO_4$, mixed, and allowed to cool. Fatty acids were then extracted twice with 10 ml of light petroleum (b.p. 40–60 °C), and extracts were pooled in glass scintillation vials and evaporated. The remaining aqueous phase was neutralized with 20% KOH, refrigerated for 1 h, then centrifuged briefly at 1000 ***g***. A portion of the supernatant was transferred to glass scintillation vials and dried under N_2 at 60–70 °C. Ethanol (1 ml) was added to ensure complete removal of water. Scintillation cocktail (10 ml; Optiphase-Safe, LKB Instruments) and 0.5 ml of water were then added to each sample, and radioactivity was measured in an LKB 1215 scintillation counter programmed to yield d.p.m. of ^3H and ^{14}C. Counting efficiency was 25% for ^3H and 50% for ^{14}C. The minimum ^3H count per sample (glycerol or fatty acids was approx. 500 d.p.m.

In some rats (lactating and with pups removed), a portion of adipose tissue was not frozen, but was transferred to Krebs–Henseleit (1932) saline at 37 °C. The tissue was carefully cut into pieces of about 10 mg and divided into 50–100 mg portions, care being taken not to let it dry. After brief blotting on tissue paper, samples were transferred into plastic flasks each containing 4 ml of Krebs–Henseleit saline (pH 7.4) with the following additions: 0.5 mCi of ^3H_2O/ml; 3% (w/v) defatted bovine serum albumin; 5.6 mM-glucose; 100 μunits of insulin/ml and 10 μM-isoprenaline (where indicated). The flasks were gassed with O_2/CO_2 (19:1) and incubated at 37 °C for 0–60 min in a metaboilc shaker. The reaction was stopped by addition of 400 μl of 2 M-H_2SO_4. Medium was separated from the tissue and frozen for determination of radioactivity and glycerol concentration (Eggstein & Kreutz, 1966). The tissue pieces were transferred to test tubes and processed as described above for experiments *in vivo*, except that the lipid extract was washed an extra time with methanol/water. The choice of adipose-tissue pieces (rather than isolated adipocytes) was dictated by the limited amount of tissue available in lactating animals. Separate experiments demonstrated linearity with time of the rates of triacylglycerol-glycerol and -acyl-group synthesis for up to 60 min in the presence or absence of isoprenaline (results not shown).

The rate of cycling was calculated as described in Brooks *et al.* (1982). The triacylglycerol fatty acid synthesis *de novo* was calculated by dividing ^3H radioactivity of the light-petroleum extract by (specific radioactivity of $^3H_2O \times 13.3$) (Windmueller & Spaeth, 1966; Jungas, 1968). Similarly, triacylglycerol-glycerol synthesis (μmol) was calculated from the ^3H radioactivity of the aqueous sample (corrected for varying recovery by [^{14}C]glycerol) divided by (specific radioactivity $\times$ 3.3) (Jungas, 1968). In the experiments with isolated tissue pieces, the radioactivity present in lipid and aqueous phases of zero-time samples was deducted. The cycling rate is then given by: 3 $\times$ (triacylglycerol-glycerol) synthesis rate − (triacylglycerol-fatty acid) synthesis rate, i.e. total esterification rate minus esterification rate of fatty acids synthesized *de novo* (Brooks *et al.*, 1982). Results are expressed per g wet wt. of tissue.

^3H_2O was purchased from Amersham International and [^{14}C]glycerol from New England Nuclear. Florisil (100–200 mesh) was from BDH. Pentobarbital, Tyloxapol, DL-isoprenaline and fatty acid-free bovine serum albumin (A-6003, batches 64F-9390, and 114F-9391)

Table 1. Effects of lactation and removal of pups from lactating animals on the rates of triacylglycerol-glycerol and triacylglycerol-fatty acid synthesis and rate of triacylglycerol/fatty acid substrate cycling in parametrial adipose tissue of rats *in vivo*

The last rate was calculated as described in the text. Results are presented as means ± S.E.M., with numbers of separate animals given in parentheses. Statistical significance is indicated as follows: *$P < 0.05$, **$P < 0.01$ versus virgin group; †$P < 0.01$ versus lactating group.

		Rates (μmol/h per g wet wt.)		
		Triacylglycerol-glycerol	Triacylglycerol-fatty acid	Triacylglycerol/fatty acid cycling
Virgin	(5)	0.91 ± 0.18	0.15 ± 0.06	2.57 ± 0.47
Virgin, starved 24 h	(6)	1.23 ± 0.04	0.04 ± 0.004	3.66 ± 0.12
Virgin, Tyloxapol treatment	(4)	1.69 ± 0.44	0.07 ± 0.02	4.98 ± 1.32
Lactating	(9)	0.029 ± 0.08*	0.06 ± 0.02	0.81 ± 0.23**
Pups removed	(7)	2.63 ± 0.92†	0.80 ± 0.27*†	7.09 ± 2.49†
Pups removed, Tyloxapol treatment	(4)	3.14 ± 0.96*†	1.16 ± 0.42*†	8.26 ± 2.52*†

were obtained from Sigma. Insulin was obtained from Novo (Actrapid MC, U-100). Biochemicals used in the assay of glycerol and triacylglycerol concentrations were supplied by Boehringer Mannheim.

Evaluation of statistical significance was carried out by the Wilcoxon method for the unpaired case.

RESULTS AND DISCUSSION

In all groups, the rates of triacylglycerol-glycerol production (total esterification rate) were substantially larger than those of synthesis *de novo* of triacylglycerol fatty acid (Table 1). In fed virgin rats, the rate *in vivo* of triacylglycerol/fatty acid cycling in white adipose tissue (2.57 ± 0.47 μmol of fatty acids re-esterified/h per g wet wt.) was similar to that reported by Brooks *et al.* (1982), based on experiments *in vitro* (Table 1). Starvation (24 h) had no effects on the rate of triacylglycerol-fatty acid or triacylglycerol-glycerol synthesis or on the rate of cycling (Table 1).

In lactating animals, the rate of triacylglycerol/fatty acid cycling was decreased to about one-third ($P < 0.02$; Table 1). This was brought about mainly by a marked decrease in the rate of triacylglycerol-glycerol synthesis ($P < 0.02$).

Removal of pups resulted in a 9-fold increase in the rate of triacylglycerol-glycerol synthesis; triacylglycerol-fatty acid synthesis rates increased even more dramatically (Table 1); and the rate of cycling was increased about 9-fold ($P < 0.01$ versus lactating group, Table 1).

The method for measurement of the cycling rates assumes a negligible esterification of exogenous (dietary or hepatic origin) fatty acids (Brooks, 1981); this assumption was shown to be valid by use of Tyloxapol. As expected, this caused dramatic increases in plasma triacylglycerol concentrations (virgin rats 4.34 ± 0.36 mM, mothers with pups removed 4.47 ± 1.07 mM, versus 0.29 ± 0.14 mM in untreated virgin rats; means $\pm$ S.E.M.). However, neither the synthesis rate of triacylglycerol glycerol nor the cycling rate were decreased (Table 1). This suggests that the rate of esterification of exogenous fatty acids is low under the conditions of the present experiment, and consequently it does not affect the rate of cycling. It can be calculated from the uptake into parametrial adipose tissue of ^{14}C-labelled fatty acids derived from exogenous triacylglycerol (Scow *et al.*, 1977; do Nascimento & Williamson, 1986) that 11–31% of the observed rate of triacylglycerol-glycerol synthesis (Table 1) can be accounted for by esterification of plasma-derived fatty acid. However, in these experiments *in vivo* the entry rate of triacylglycerol into the plasma was increased 2–3-fold, and therefore the calculated values are likely to be overestimates of the possible contribution of exogenous fatty acid.

In vitro, under basal conditions (insulin 100 μunits/ml) the rate of glycerol release was higher in tissue obtained from lactating rats than in tissue from lactating rats which had the pups removed; this difference was abolished when lipolysis was maximally stimulated by 10 μM-isoprenaline (Table 2).

The basal rate of triacylglycerol/fatty acid cycling in incubated adipose tissue from lactating rats was higher than *in vivo* (cf. Table 1), and was similar to that in tissue incubated *in vitro* from animals with litters removed (Table 2). In tissue from this latter group, isoprenaline increased the cycling rate 5-fold (similarly to previous

Table 2. Synthesis rates *in vitro* of triacylglycerol glycerol and fatty acid, rates of triacylglycerol/fatty acid substrate cycling, glycerol release and percentage re-esterification of fatty acids

Adipose-tissue pieces were incubated for 1 h in the presence of insulin (100 μunits/ml) with or without isprenaline (10 μM). Synthesis, cycling and release rates are all given in μmol/h per g wet wt. Each value is the mean $\pm$ S.E.M. for three animals.

Condition of animal	Additions to incubation medium	Rate (μmol/h per g wet wt.)				Fatty acids re-esterified (%)
		Glycerol release	Triacylglycerol glycerol	Triacylglycerol fatty acid	Triacylglycerol/fatty acid substrate cycling	
Lactating	Insulin	1.62 ± 0.51	0.80 ± 0.12	< 0.02	2.40 ± 0.35	56.5 ± 13.1
	Insulin + isoprenaline	6.11 ± 0.56	0.34 ± 0.13	< 0.02	1.01 ± 0.38	6.0 ± 2.5
Lactating and pups removed	Insulin	0.76 ± 0.27	0.68 ± 0.22	0.21 ± 0.07	1.83 ± 0.59	76.8 ± 1.9
	Insulin + isoprenaline	7.59 ± 0.14	3.37 ± 0.52	0.15 ± 0.06	9.97 ± 1.49	43.9 ± 5.6

findings in male rats; Brooks *et al.*, 1982). In the tissue from lactating rats, isoprenaline decreased the cycling rate by more than 50% (Table 2). These effects of isoprenaline were brought about by changes in the rate of triacylglycerol-glycerol synthesis; rates of synthesis of fatty acid were not appreciably affected by the drug, although basal rates differed considerably between lactating and litter-removed groups (Table 2) (cf. Agius *et al.*, 1979). Catecholamines may increase glycerol 3-phosphate availability for triacylglycerol-glycerol synthesis via their stimulation of glucose uptake and glycolytic flux (Halperin & Denton, 1969; Saggerson & Greenbaum, 1970), and this effect may be impaired in adipose tissue from lactating rats.

The absence of β-adrenergic stimulation of the cycling rate in adipose tissue *in vitro* during lactation is an interesting finding (cf. Brooks, 1981; Brooks *et al.*, 1982) and confirms some observations by Smith & Walsh (1976), who measured glycerol and non-esterified fatty acid release. It cannot be explained by a general desensitization to catecholamines, since lipolysis (measured as glycerol release) is stimulated, albeit relatively less than after removal of pups (see also Smith & Walsh, 1976). The esterification pathway appears to be desensitized to catecholamines (see Cubero *et al.*, 1983), which would ensure that adrenergic stimulation would not deprive mammary gland of lipid. The percentage of fatty acid re-esterified [calculated as (cycling rate $\times$ 100)/(3 $\times$ rate of glycerol release)] was lower in the lactating group under basal conditions, and was substantially decreased by isoprenaline. However, in rats from which the pups had been removed, even a high concentration of isoprenaline did not decrease re-esterification below about 40% (Table 2).

During lactation, there are decreases in the rate of adipose-tissue lipogenesis (Agius *et al.*, 1979; Flint *et al.*, 1981) and lipid accumulation mediated by lipoprotein lipase (Hamosh *et al.*, 1970; Scow *et al.*, 1977; do Nascimento & Williamson, 1986), but the rate of lipolysis is increased. These changes ensure direction of lipid fuels away from adipose tissue to the mammary gland. The low plasma insulin concentration (Robinson *et al.*, 1978; Flint *et al.*, 1979; Burnol *et al.*, 1983; Jones *et al.*, 1984) and the low rate of fatty acid esterification (Cubero *et al.*, 1983) probably contribute to this change in direction of metabolism. It thus seems reasonable to assume that the rate of cycling should also decrease, so as not to impair the flux of lipid energy to the lactating gland. However, the simple notion that cycling rate is inversely related to lipolytic rate is not supported by the findings in starved animals, where cycling is maintained at the same rate as in controls. These findings support the view that a major role of substrate cycling is to improve the sensitivity in metabolic regulation (Crabtree & Newsholme, 1985); the increased mobilization of lipid in short-term starvation must be readily reversed on re-feeding, and such a high sensitivity of metabolic regulation could be achieved via substrate cycling. By contrast, during lactation the major role of adipose tissue is to provide lipid, so that, despite an increase in dietary intake, a high sensitivity is not required in this tissue at this time. Thus the differing cycling rates can be viewed in terms of providing different degrees of sensitivity to various regulators.

Lipogenesis from glucose results in a net production of ATP (Flatt, 1970), so that the process of lipogenesis may be limited under some conditions by ATP accumulation. Brooks (1981) has proposed that such an accumulation could be prevented by the triacylglycerol/fatty acid cycle. In the present experiment (assuming that all synthesized fatty acid is recovered in triacylglycerol) the mean increase in lipogenesis *in vivo* after removal of pups (0.74 μmol/h per g wet wet.) would mean an 'excess' ATP production of 11 μmol/h per g wet wt. (Flatt, 1970; Brooks, 1981). The increase in cycling rate (6.28 μmol/h per g wet wt.) would result in an ATP consumption of about 21 μmol/h per g wet wt. (assuming $3\frac{1}{3}$ molecules of ATP hydrolysed/molecule of re-esterified fatty acid; Brooks, 1981).

The decreased cycling rate in lactation also serves to conserve energy. The decreased energy expenditure could amount to about 150 J/day in a 300 g rat (cf. Brooks, 1981). Although this in itself might seem insignificant, other factors, such as decreased thermogenesis in brown adipose tissue (Trayhurn *et al.*, 1982) and decreased lipid oxidation (do Nascimento & Williamson, 1986), also contribute to increased energy efficiency. Studies on the change in metabolic rate in response to a test meal or noradrenaline infusion in human lactation have suggested that the non-lactational component of energy expenditure is decreased (Illingworth *et al.*, 1986). This would be advantageous if food supplies are limited.

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