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RESEARCH ARTICLE

Extending food deprivation reverses the short-term lipolytic response to fasting: role of the triacylglycerol/fatty acid cycle

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SUMMARY

The effects of short-term food deprivation on lipid metabolism are well documented, but little is known about prolonged fasting. This study monitored the kinetics of glycerol (rate of appearance, $\dot{R}_a$ glycerol) and non-esterified fatty acids ($\dot{R}_a$ NEFA) in fasting rabbits. Our goals were to determine whether lipolysis is stimulated beyond values seen for short-term fasting, and to characterize the roles of primary (intracellular) and secondary (with transit through the circulation) triacylglycerol/fatty acid cycling (TAG/FA cycling) in regulating fatty acid allocation to oxidation or re-esterification. $\dot{R}_a$ glycerol (9.62±0.72 to 15.29±0.96 μmol kg^{-1} min^{-1}) and $\dot{R}_a$ NEFA (18.05±2.55 to 31.25±1.93 μmol kg^{-1} min^{-1}) were stimulated during the first 2 days of fasting, but returned to baseline after 4 days. An initial increase in TAG/FA cycling was followed by a reduction below baseline after 6 days without food, with primary and secondary cycling contributing to these responses. We conclude that the classic activation of lipolysis caused by short-term fasting is abolished when food deprivation is prolonged. High rates of re-esterification may become impossible to sustain, and TAG/FA cycling could decrease to reduce its cost to 3% of total energy expenditure. Throughout prolonged fasting, fatty acid metabolism gradually shifts towards increased oxidation and reduced re-esterification. Survival is achieved by pressing fuel selection towards the fatty acid dominance of energy metabolism and by slowing substrate cycles to assist metabolic suppression. However, TAG/FA cycling remains active even after prolonged fasting, suggesting that re-esterification is a crucial mechanism that cannot be stopped without harmful consequences.

Key words: lipid kinetics, lipolysis, substrate cycling.

INTRODUCTION

Metabolic suppression and changes in fuel selection are complementary strategies to cope with prolonged fasting (Cahill, 1970; Markussen and Oritsland, 1986; Merkt and Taylor, 1994). When feeding stops, a shift to the predominant use of lipids is essential to avoid the depletion of small carbohydrate reserves and spare structural proteins (McCue, 2010; Weber, 2011). Therefore, lipolysis and the oxidation of non-esterified fatty acid (rate of oxidation, $\dot{R}_{ox}$ NEFA) are both stimulated, although current information on lipid kinetics during fasting only comes from short-term studies in humans (Carlson et al., 1994; Elia et al., 1987; Klein et al., 1993; Wolfe et al., 1987b). After 3–4 days without feeding, a 2.5-fold increase in lipolytic rate is observed, typically measured as the rate of appearance of glycerol ($\dot{R}_a$ glycerol) or NEFA ($\dot{R}_a$ NEFA). Together with lipolytic activation, increments of 60–160% in $\dot{R}_{ox}$ NEFA have been documented for a variety of mammals (Carlson et al., 1994; Reidy and Weber, 2004; Weber and O'Connor, 2000). All these adjustments are responsible for increasing the relative contribution of lipid oxidation to total energy expenditure from post-absorptive values below 46% to 75–94% after a 3 day fast. It is unknown whether longer periods of food deprivation would amplify these responses, or reverse them to promote metabolic suppression. In this study, we chose to characterize the response of smaller mammals than humans (3 kg rabbits) deprived of food for a longer period (6 days), to investigate the effects of a stronger fasting stress than in all previous studies of lipid kinetics.

Fatty acids made available by lipolysis can either be oxidized or returned to storage through re-esterification. Simultaneous lipolysis and re-esterification form the triacylglycerol/fatty acid cycle (TAG/FA cycle), an important substrate cycle for the regulation of lipid metabolism (Reidy and Weber, 2002; Wolfe et al., 1990). In humans, the TAG/FA cycle plays a crucial role in modulating the availability of fatty acids for energy metabolism during prolonged exercise (Wolfe et al., 1990). However, its involvement in controlling lipid supply to working muscles in long-distance migrants has not been investigated by comparative physiologists. Similarly, the stimulation of the TAG/FA cycle is an established mechanism for thermogenesis during cold exposure (Vallerand et al., 1999) and when leptin levels are elevated (Reidy and Weber, 2002). To date, however, the potential contribution of this substrate cycle to heat production has not been evaluated in hibernators. The synchronized measurement of $\dot{R}_a$ glycerol, $\dot{R}_a$ NEFA and $\dot{R}_{ox}$ NEFA allows us not only to quantify total TAG/FA cycling but also to distinguish between primary cycling (re-esterification without exit from intracellular sites of lipolysis) and secondary cycling (re-esterification after transit through the circulation) (Campbell et al., 1992; Wolfe et al., 1990). Post-absorptive mammals support high baseline rates of TAG/FA cycling because 70–85% of the fatty acids provided by lipolysis are re-esterified (McClelland et al., 2001; Reidy and Weber, 2002; Wolfe et al., 1990). In humans, the TAG/FA cycle is further stimulated during fasting. However, whether secondary cycling participates in this response remains unknown

because previous studies only dealt with changes in primary cycling (Wolfe et al., 1987b) or total cycling (Elia et al., 1987; Klein et al., 1989). Paradoxically, substrate cycles do consume ATP (Newsholme and Crabtree, 1976), and it would therefore appear counterintuitive to stimulate TAG/FA cycling during food deprivation, when saving energy becomes essential for survival.

Long-distance migrants and hibernators are among the champions of fasting and they would make excellent models to study the physiology of extreme food deprivation. However, their changes in lipid kinetics are partly caused by physiological stresses other than strictly fasting (exercise, hypothermia, metabolic suppression). In this study, we focused on the physiological response to severe food deprivation and opted to determine baseline values from a species with no particular adaptation for fasting. Therefore, we quantified changes in the glycerol and NEFA kinetics of rabbits deprived of food for 6 days. Our first goal was to determine whether longer term food deprivation stimulates lipolysis beyond values observed after short-term fasting, or if metabolic suppression makes such a response unnecessary. Changes in the composition of plasma NEFA were also monitored to assess whether particular fatty acids would be mobilized preferentially. Our second goal was to characterize the roles of primary and secondary TAG/FA cycling in regulating the relative allocation of fatty acids to oxidation or re-esterification. We hypothesized that prolonged fasting would: (i) increase lipolysis and fatty acid oxidation beyond short-term values to promote the use of abundant lipid reserves, and (ii) reduce re-esterification to decrease the energy cost of operating the TAG/FA cycle.

MATERIALS AND METHODS

Animals and surgery

All experimental procedures were approved by the Animal Care Committee of the University of Ottawa in accordance with the requirements of the Canadian Council on Animal Care. Adult New Zealand white rabbits (*Oryctolagus cuniculus* L.) of both sexes (body mass 2.96±0.11 kg, ~13 weeks; Charles River Laboratories, Montreal, QC, Canada) were used for *in vivo* lipid kinetics and analyses of lipid reserves. Previous work on fasting-induced changes in fuel metabolism for New Zealand White rabbits of the same mass/age showed no gender differences (Reidy and Weber, 2004). The animals were fed standard rabbit chow (52% carbohydrate, 3% lipid, 16% protein, 14% crude fiber and 15% water) and always had access to drinking water *ad libitum*. Daily food and water intake was recorded for 3 days before surgery to establish baseline values. Subcutaneous injections of buprenorphine (0.02 mg kg^{-1}) were given before and after catheterization to eliminate surgical discomfort. Animals fasted for 12 h were catheterized under isoflurane anesthesia, after intramuscular injection of ketamine (15 mg kg^{-1}), midazolam (0.5 mg kg^{-1}) and robinel (0.005 mg kg^{-1}). Polyethylene catheters were placed in the right jugular vein and right carotid artery (Intramedic PE-50, Becton Dickinson, Sparks, MD, USA). They were inserted 7 cm towards the heart and filled with saline containing 40 U ml^{-1} heparin and 125,000 U ml^{-1} penicillin G. The catheters were sutured to the vessels, tunneled under the skin and exteriorized between the scapulas. They were flushed daily with pure saline, taking special care to withdraw the heparinized saline before flushing. Experiments were started 3–5 days after surgery, when rates of food and water consumption had returned to pre-surgery levels for at least 2 days. Some animals were not catheterized; they were randomly divided in two groups (fed controls or 6 day fast) and used to evaluate the effects of food deprivation on adipose reserves and organ size.

Fasting protocol and indirect calorimetry during measurement of metabolite kinetics

Each animal was placed individually in a closed respirometer (54×38×67 cm) to monitor the rates of O_2 consumption ($\dot{M}_{O_2}$) and CO_2 production ($\dot{M}_{CO_2}$) using a calibrated Oxymax respirometry system (Columbus Instruments, Columbus, OH, USA) (for details, see Fournier and Weber, 1994). Gas exchange measurements were carried out continuously throughout the experiments, except for 30 min per day to collect urine (for measuring nitrogen excretion), to clean the respirometer and to calibrate the gas analyzers. The respirometer was supplied with air at 3–8 l min^{-1} through a mass flow regulator accurate within 1% of full scale as calibrated with a reference volume meter (Porter Instruments, Hatfield, PA, USA). A small fan, enclosed in the respirometer lid, ensured that gases were continuously mixed. The O_2 and CO_2 analyzers were calibrated once a day with known gas mixtures. $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ were corrected for dry gas under standard temperature and pressure (STP) conditions. Drinking water was available *ad libitum* at all times. Food was available *ad libitum* for the first 3 days, before the animals were fasted either for 6 days or until they had lost 15% of initial body mass (a limit set by our institutional Animal Care Committee). Experiments only had to be interrupted for one individual that reached 15% mass loss after 5 days of fasting. Therefore, the results for metabolic measurements have a sample size of 6, except for the last day of fasting when N=5.

Fatty acid and glycerol kinetics

Every 2 days, glycerol and fatty acid kinetics were measured by continuous infusion of [2-^{3}H]glycerol (292.3±11.8 kBq ml^{-1}) and [1-^{14}C]palmitate (310.4±20.4 kBq ml^{-1}) (Amersham, Oakville, ON, Canada) for 1 h at 5 ml h^{-1} through the venous catheter using a calibrated syringe pump (Harvard Apparatus, South Natick, MA, USA). These conditions ensured that isotopic steady state was reached within 30 min of infusion. The infusate was freshly prepared for each infusion by mixing labeled palmitate with a saline solution of delipidated rabbit albumin and by adding labeled glycerol. Palmitate and glycerol (labelled+unlabelled) were infused in trace amounts accounting for <0.15% of the endogenous rates of appearance measured in fed animals. Measurement of glycerol and fatty acid fluxes was performed in fed animals, and after 2, 4 and 6 days of fasting. Blood samples (1 ml each) were drawn from the arterial catheter 40, 45, 50 and 55 min after the start of each infusion. Plasma was separated immediately, stored at –20°C, and analyzed within 7 days.

Sample analyses

Urinary nitrogen content was measured using the Kjeldahl method (Weber and O'Connor, 2000). For plasma analyses, preliminary experiments showed that plastic tubes adsorb significant amounts of glycerol. We determined that the loss of glycerol radioactivity could be minimized to <2% by using glass tubes pre-treated with a solution of unlabeled glycerol to saturate adsorption sites. Plasma lipids were separated from water-soluble metabolites using chloroform:methanol (2:1 v/v) (Folch et al., 1957). Aqueous and organic phases were dried on a rotating evaporator (Büchi Rotavapor, Flawil, Switzerland). The aqueous phase was resuspended in ethanol:water (1:1 v/v) and the organic phase in hexane:isopropanol (3:2 v/v). The concentration and radioactivity of palmitate, total NEFA and glycerol were measured as described previously (Bernard et al., 1999; McClelland et al., 1995). NEFA were separated from other plasma lipids by sequential elution using Supelclean solid-phase extraction tubes (100 mg LC-NH2, Sigma,

St Louis, MO, USA) (Bernard et al., 1999; Maillet and Weber, 2006). All activities were measured by scintillation counting on a Tri-Carb 2500 (Canberra Packard, Mississauga, ON, Canada) using external quench correction. Plasma NEFA concentrations were measured by gas chromatography after methylation (Tserng et al., 1981), using heptadecanoic acid as an internal standard. The concentration of individual fatty acids was quantified on a Hewlett-Packard 5890 series II gas chromatograph with flame-ionization detector and a 30 m fused silica column (DB-23; J&W Scientific, Folsom, CA, USA) as described previously (McClelland et al., 1999). Exact retention times of individual fatty acids were determined with pure standards (Sigma).

Calculations and statistics

Rates of lipid oxidation ($\dot{R}_{ox}$ NEFA) were calculated from $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$, and were corrected for protein oxidation (computed from the rate of nitrogen excretion) as recommended elsewhere (Frayn, 1983). The use of Frayn's equations to calculate total lipid oxidation at the whole organism level has been thoroughly validated and this approach has been applied successfully in wide-ranging human and animal studies (Ferrannini, 1988; Frayn, 1983; Livesey and Elia, 1988). $\dot{R}_a$ glycerol and $\dot{R}_a$ palmitate were calculated using the steady-state equation of Steele (Steele, 1959). For each tracer infusion (i.e. each fasting time), four blood samples were taken to ensure that isotopic steady state had been reached. The metabolite concentrations and radioactivities for 0, 2, 4 and 6 days of food deprivation were calculated by averaging measurements from the four samples measured at each fasting time. $\dot{R}_a$ palmitate was divided by the fractional contribution of palmitate to total plasma NEFA to calculate $\dot{R}_a$ NEFA. Total, primary and secondary TAG/FA cycling were calculated from $\dot{R}_a$ glycerol, $\dot{R}_a$ NEFA and $\dot{R}_{ox}$ NEFA as follows:

$$\text{Total TAG/FA cycling} = (3 \times \dot{R}_a \text{ glycerol}) - \dot{R}_{ox} \text{ NEFA}, \quad (1)$$

$$\text{Primary TAG/FA cycling} = (3 \times \dot{R}_a \text{ glycerol}) - \dot{R}_a \text{ NEFA}, \quad (2)$$

$$\text{Secondary TAG/FA cycling} = \dot{R}_a \text{ NEFA} - \dot{R}_{ox} \text{ NEFA}. \quad (3)$$

Calculation of primary and secondary TAG/FA cycling using these equations has been widely used in human studies (Bahr et al., 1990; Elia et al., 1987; Romijn et al., 1993; Vallerand et al., 1999; Wolfe et al., 1987a; Wolfe et al., 1990) and in comparative physiology (Kalderon et al., 2000; McClelland et al., 2001; Reidy and Weber, 2002; Weber et al., 1993). The equations were derived from the fact that glycerol and fatty acids released by lipolysis do not behave similarly. The total amount of fatty acids made available by lipolysis from adipose reserves is equal to $3\times\dot{R}_a$ glycerol because this tissue lacks glycerokinase. Therefore, all the glycerol produced by lipolysis must come out of the cell before it is further metabolized. This is not the case for fatty acids because they can be re-esterified within the same cell where they have been hydrolyzed from TAG. A detailed discussion of the assumptions and limitations associated with this common approach can be found in the following references (Reidy and Weber, 2002; Wolfe and Chinkes, 2005; Wolfe et al., 1987a; Wolfe et al., 1990). The energy cost of TAG/FA cycling was calculated by assuming that the re-esterification of each TAG requires 8 ATP (or 602 kJ mol^{-1}). Oxygen consumption was converted to energy expenditure to calculate the relative cost of cycling (0.45 J µmol^{-1} O_2). Statistical comparisons were performed using repeated measures ANOVA with the Bonferroni *post hoc* test to determine which means were different from baseline. Percentages were transformed to the arcsine of their square root before analyses. All values presented are means ± s.e.m., and the significance threshold was set at $P<0.05$.

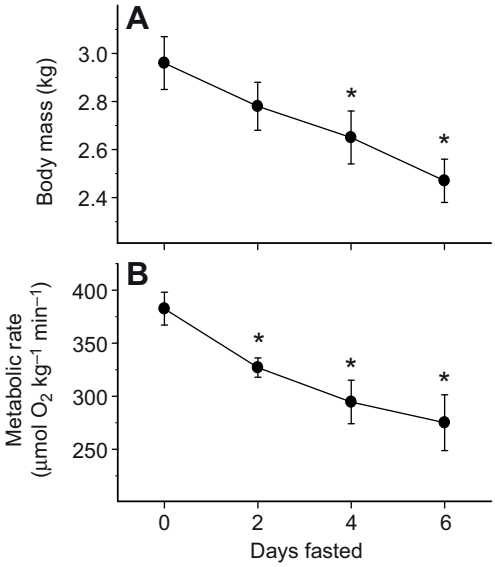

Fig. 1. Changes in body mass (A) and mean 24 h metabolic rate ($\dot{M}_{O_2}$) of adult New Zealand white rabbits (B) during a 6 day fast (N=6, except day 6 when N=5). *Significant difference from fed controls (P<0.05).

RESULTS

Body mass and metabolic rate

The 6 day fasting period caused a decrease in body mass (Fig. 1A; P<0.05) and in mass-specific metabolic rate (Fig. 1B; P<0.05). Carcass analyses of a separate group of animals showed that the change in body mass was mainly caused by a large decrease in adipose tissue mass (Table 1; P<0.05), and by declines in skeletal muscle, liver and heart mass (P<0.05).

Glycerol and fatty acid metabolism

Large increases in plasma glycerol concentration (Fig. 2A) and $\dot{R}_a$ glycerol (from 9.62±0.72 to 15.29±0.96 µmol kg^{-1} min^{-1}; Fig. 2B) were observed after 2 days of fasting (P<0.05). However, glycerol concentration and glycerol flux returned to the baseline values measured in the post-absorptive state when fasting was extended to 4–6 days (Fig. 2; P>0.05). Similarly, plasma NEFA concentration (Fig. 3A) and $\dot{R}_a$ NEFA (18.05±2.55 to 31.25±1.93 µmol kg^{-1}min^{-1};

Table 1. Absolute and relative tissue masses of adult New Zealand White rabbits

	Fed	Fasted
Body mass (kg)	3.633±0.088	2.624±0.063*
Adipose tissue (g)	649.1±68.7	102.8±2.8*
% total body mass	17.85±1.80	4.11±0.08*
Skeletal muscle (g)	1439.2±30.8	1086.4±64.5*
% lean body mass	48.23±0.70	43.09±2.42*
Heart (g)	11.5±0.7	6.6±0.9*
% lean body mass	0.35±0.02	0.27±0.04*
Liver (g)	96.2±10.4	47.6±5.8*
% lean body mass	3.21±0.27	2.00±0.32*

A group of fed animals (control) is compared with a treatment group fasted for 6 days.
Values are means ± s.e.m. (N=3).
*Significant reduction (P<0.05).

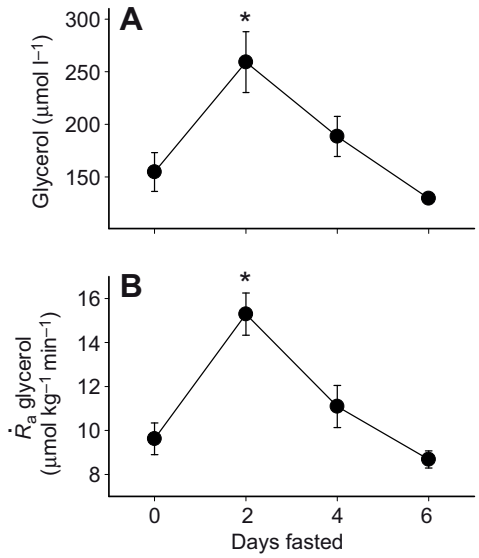

Fig. 2. Changes in plasma glycerol concentration (A), and rate of appearance of glycerol or lipolytic rate ($\dot{R}_a$ glycerol; B) in adult rabbits during a 6 day fast (N=6, except day 6 when N=5). *Significant difference from fed controls (P<0.05).

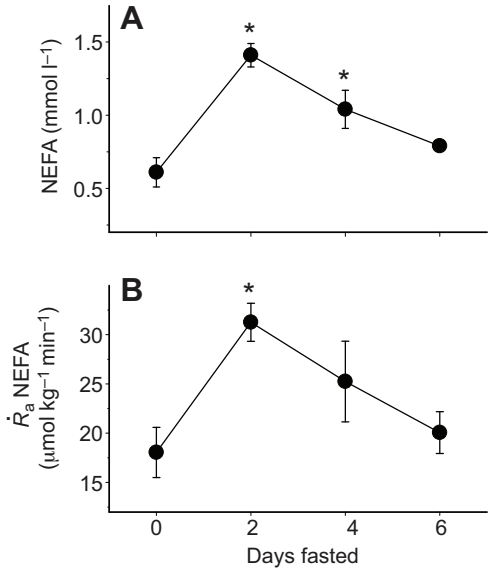

Fig. 3. Changes in the concentration of plasma non-esterified fatty acids (NEFA; A) and of their rate of appearance ($\dot{R}_a$ NEFA; B) in adult rabbits during a 6 day fast (N=6, except day 6 when N=5). *Significant difference from fed controls (P<0.05).

Fig. 3B) were strongly stimulated after 2 days of fasting (P<0.05), but both parameters returned to baseline when fasting was prolonged for 4–6 days (Fig. 3; P>0.05). The effects of fasting on plasma NEFA composition are reported in Table 2. Only six fatty acids had a relative abundance >3%: palmitate (16:0, expressing the number of carbons:number of double bonds), stearate (18:0), palmitoleate (16:1), oleate (18:1), linoleate (18:2) and linolenate (18:3). Together, saturated fatty acids (16:0 and 18:0) always accounted for more than 50% of total NEFA. Two days of fasting caused a large decrease in the percentage of 18:0, but an increase in the percentage of 18:1 and 18:2 (P<0.05). In contrast to the responses seen for $\dot{R}_a$ NEFA and absolute NEFA concentration (Fig. 3), changes in NEFA composition were not reversed as fasting was prolonged to 6 days (Table 2).

Metabolic fate of fatty acids: oxidation *vs* re-esterification

Fig. 4 shows the effects of prolonged fasting on the partitioning between fatty acid oxidation and re-esterification. During these experiments, fatty acid flux through re-esterification was always at least twice as high as through oxidation. This was true not only for absolute rates (expressed in μmol fatty acids $kg^{-1} min^{-1}$; Fig. 4A) but also for relative rates (expressed as a percentage of total fatty acids released by lipolysis or $3\times\dot{R}_a$ glycerol; Fig. 4B). The absolute rate of fatty acid oxidation doubled after 2 days of fasting (P<0.05) and stayed elevated until the end of the experiments. The absolute rate of re-esterification increased above baseline after 2 days of fasting (P<0.05), returned to baseline after 4 days of fasting (P>0.05) and decreased significantly below baseline after 6 days of fasting (P<0.05; Fig. 4A). Prolonged fasting caused a progressive increase in the relative rate of oxidation (from 12% to 33%) and a progressive decrease in the relative rate of re-esterification (from 88% to 67%) (Fig. 4B).

The percentage of total fatty acids undergoing primary re-esterification (within the cells where lipolysis takes place) and secondary re-esterification (after transit through the circulation) is presented in Fig. 5. Secondary re-esterification (43–49%) was always higher than primary re-esterification (24–39%; P<0.05), but these relative values were not affected by prolonged fasting (P>0.05). On average, secondary re-esterification accounted for 47.4% and primary re-esterification for 29.7% of total fatty acids released by lipolysis. The energy cost of the TAG/FA cycle (or total re-esterification) is presented in Fig. 6. The absolute cost of re-esterification expressed in $J kg^{-1} min^{-1}$ increased above baseline after 2 days of fasting (P<0.05), returned to baseline after 4 days of fasting (P>0.05) and decreased below baseline after 6 days of fasting (P<0.05) (Fig. 6A). The relative cost of re-esterification expressed as a percentage of total energy expenditure increased after 2 days of fasting, but returned to baseline when fasting was prolonged (Fig. 6B).

Table 2. Changes in plasma NEFA composition during 6 days of fasting

Days fasted	16:0	18:0	16:1	18:1	18:2	18:3
0	28.3±1.6	31.1±2.5	2.0±0.4	13.9±0.9	12.7±0.9	7.1±2.2
2	28.5±1.4	21.6±2.4*	3.0±0.7	19.3±1.2*	19.8±2.1*	6.1±1.1
4	28.2±2.2	24.2±2.4*	2.1±0.2	18.0±0.8*	16.3±1.6*	7.0±1.8
6	30.5±1.1	23.9±2.4*	2.0±0.4	18.2±1.2*	18.3±1.5*	4.1±1.0

NEFA, non-esterified fatty acid. Fatty acids are identified as X:Y where X is the number of carbons and Y the number of double bonds. Fatty acids accounting for less than 1% were not included. Main plasma fatty acids are palmitate (16:0), stearate (18:0), palmitoleate (16:1), oleate (18:1), linoleate (18:2) and linolenate (18:3).

Values are % of total non-esterified fatty acids by mass (means ± s.e.m.; N=6, except day 6 when N=5).

*Significant difference from fed controls measured on day 0 (P<0.05).

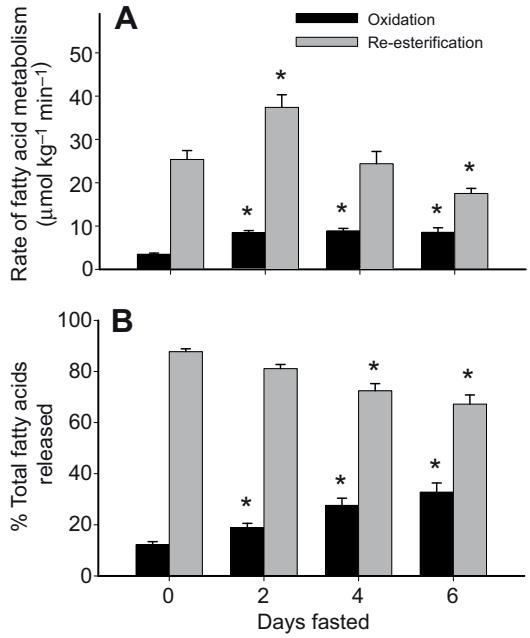

Fig. 4. Changes in absolute (A) and relative (B) rate of fatty acid oxidation and fatty acid re-esterification for adult rabbits during a 6 day fast (N=6, except day 6 when N=5). *Significant difference from fed controls (P<0.05).

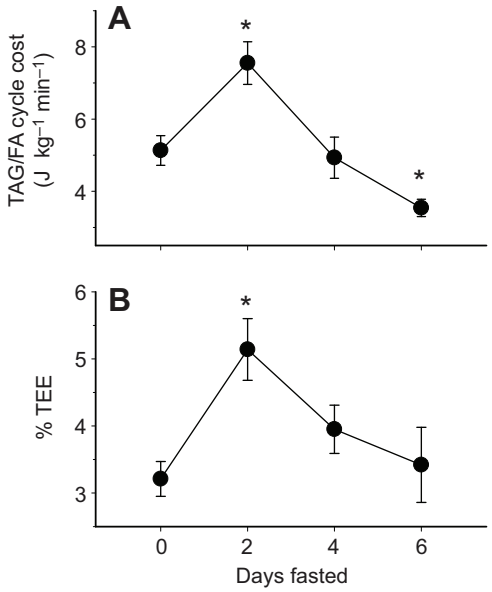

Fig. 6. Effects of prolonged fasting on the energy cost of the triacylglycerol/fatty acid (TAG/FA) cycle in adult rabbits. (A) Absolute values. (B) Relative values expressed as a percentage of total energy expenditure (%TEE) (N=6, except day 6 when N=5). *Significant difference from control values in fed animals (P<0.05).

DISCUSSION

This study shows that lipolysis is temporarily stimulated at the onset of fasting, but returns to baseline with prolonged food deprivation. In rabbits, rates of glycerol and fatty acid release from adipose reserves are strongly stimulated during the first 2 days of fasting, but they return to post-absorptive levels after 4 days without food. The results also reveal that both primary and secondary fatty acid cycling contribute to the increase in re-esterification seen at the onset of fasting. After this early response, TAG/FA cycling becomes inhibited below baseline with extended fasting. By contrast, the initial stimulation of fatty acid oxidation persists, even after a 6 day fast that causes deep metabolic suppression. Together, these coordinated changes gradually decrease the relative allocation of fatty acids to re-esterification and increase their relative contribution to oxidation. Rabbits respond to 6 days of fasting: (a) by suppressing metabolism, (b) by modulating lipolysis accordingly to ensure that most of the needs for oxidative fuel are covered by large adipose reserves, and (c) by maintaining an active TAG/FA cycle of low energy cost.

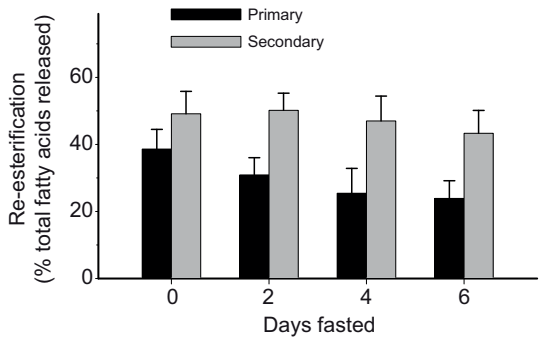

Fig. 5. Proportion of total re-esterified fatty acids undergoing primary and secondary re-esterification in adult rabbits during a 6 day fast. In the primary pathway, fatty acids are re-esterified within the cells where they have been made available by lipolysis. In the secondary pathway, fatty acids transit through the circulation before being re-esterified (N=6, except day 6 when N=5).

Activation of lipolysis is temporary

All previous information on lipid kinetics during food deprivation was obtained from 70 kg humans that were fasted for up to 4 days. This physiological stress was met by a 2- to 3-fold increase in lipolysis, and 5% decreases in body mass and metabolic rate (Carlson et al., 1994; Elia et al., 1987; Klein et al., 1990; Klein et al., 1993; Klein et al., 1986; Wolfe et al., 1987b). By monitoring 3 kg rabbits deprived of food for 6 days, we have measured the metabolic effects of a stronger stress than in any previous investigation of lipid kinetics during fasting. The animals showed a strong initial activation of $\dot{R}_a$ glycerol (+59%; Fig. 2) and $\dot{R}_a$ NEFA (+73%; Fig. 3) during the first 2 days without food. However, these responses were completely reversed after a 6 day fast (Figs 2–3) that caused a 14% decrease in body mass (Fig. 1A; mostly adipose tissue loss, see Table 1) and major metabolic suppression (–28% in $\dot{M}_{O_2}$; Fig. 1B). This reversal of lipolytic stimulation with prolonged fasting had not been demonstrated previously. In a review of the literature, we were only able to find three reports addressing the long-term effects of food deprivation on mammalian lipid metabolism. However, they all dealt with changes in circulating metabolite levels, not with changes in lipid kinetics. Two rabbit studies showed that early increases in the concentration of lipoproteins (Aladjem and Rubin, 1954) and cholesterol (Swaner and Connor, 1975) were maintained when fasting was prolonged for 7 and 32 days, respectively, giving no indication that lipolytic activation might be temporary. Only one study on rats provided indirect support for a potential reversal of lipolytic stimulation. It showed that the initial increases in the plasma concentrations of NEFA (+95%) and glycerol (+47%) seen after the first 3 days were not sustained when fasting was prolonged for a period of 6 days (Parrilla, 1978).

Even though lipolysis had returned to post-absorptive levels after 4–6 days of fasting (Figs 2 and 3), it continued to provide far more fatty acids than necessary to support accelerated rates of lipid oxidation (Fig. 4A). At the end of our experiments, two-thirds of the fatty acids made available by lipolysis were still channeled towards re-esterification because only one-third was needed to cover the increased oxidative needs (Fig. 4). Therefore, active re-esterification was still observed when energy reserves were being stretched to their limit, suggesting that the maintenance of high flux through the TAG/FA cycle is a physiological necessity. It is conceivable that rapid restructuring of triacylglycerol stores and membrane phospholipids through continuous fatty acid cycling could be a more important metabolic requirement than previously recognized (e.g. Magnoni et al., 2008).

Fasting causes stimulation, then inhibition of TAG/FA cycling

The results show that the TAG/FA cycle is strongly stimulated by 2 days of fasting (+47%), returns to post-absoptive levels after 4 days, but is eventually inhibited below baseline after 6 days without food (–31%; Fig. 4A). Variation in both primary and secondary cycling is responsible for causing these changes, because fasting does not cause any significant shift in their relative contribution to total re-esterification (Fig. 5). Under all conditions, rabbits support higher fluxes of secondary than primary cycling (Fig. 5) (Reidy and Weber, 2002), as previously reported for humans (Wolfe et al., 1990) but opposite to the case for rats (Kalderon et al., 2000; McClelland et al., 2001). It is unclear whether body size (or mass-specific metabolic rate) plays a role in setting these interspecific differences in the relative importance of primary *vs* secondary cycling. In the post-absorptive state, rabbits re-esterify 88% of the fatty acids released by lipolysis with 38% going to primary cycling and 50% to secondary cycling. Except for lower relative rates of primary cycling, humans show very similar values: 70% of total fatty acids are re-esterified, 20% to primary and 50% to secondary cycling (Wolfe et al., 1990).

Previous studies show that 3–4 days of fasting cause a dramatic increase in TAG/FA cycling in humans [3-fold increase in primary cycling (Wolfe et al., 1987b) or 6-fold increase in total cycling (Elia et al., 1987)]. In our experiments, 2 days of fasting also stimulated TAG/FA cycling in rabbits, although not as strongly as in humans (Fig. 4A, Fig. 6). Wolfe et al. had hypothesized that fasting-induced stimulation of TAG/FA cycling may be used as a convenient mechanism to increase glycerol availability for gluconeogenesis without flooding the circulation with fatty acids (Wolfe et al., 1987b). Because cycling is eventually decreased when fasting is prolonged (Fig. 4A), this potential requirement to supply gluconeogenesis might only be temporary, unless alternative gluconeogenic precursors can replace glycerol after the first 2 days of fasting.

Energy cost of TAG/FA cycling and metabolic suppression

Activation of substrate cycles through physiological stress has been demonstrated for a variety of organisms including humans during exercise or cold exposure (Bahr et al., 1990; Romijn et al., 1993; Vallerand et al., 1999; Wolfe et al., 1990), burn patients (Wolfe et al., 1987a), malignant-hyperthermic pigs (Clark et al., 1973b) and flying insects (Clark et al., 1973a). Substrate cycles have an energy cost that rises when they are stimulated. Therefore, the initial increase in TAG/FA cycling observed in the early stages of fasting is rather surprising because extra ATP must be spent at a time when the key to survival is energy conservation (Fig. 6). The subsequent decline in cycling seen with extended fasting may occur because accelerated re-esterification cannot be sustained. It is conceivable that inhibition of TAG/FA cycling may ultimately be required to contribute to the energy savings of metabolic suppression. However, our results provide no proof of this possibility.

The relative cost of TAG/FA cycling increases from 3 to 5% of total energy expenditure during the first 2 days of fasting (Fig. 6B), and these values are consistent with what has been reported for rats and humans (Elia et al., 1987; McClelland et al., 2001; Vallerand et al., 1999; Wolfe et al., 1987a; Wolfe et al., 1990; Wolfe and Peters, 1987). When food deprivation is further prolonged, the inhibition of absolute cycling rate (Fig. 6A) returns the relative cost of TAG/FA cycling to 3% of energy expenditure (Fig. 6B), even though total metabolic rate is now greatly depressed (Fig. 1B). Increased metabolic efficiency during and after food restriction is routinely observed in human dieters as they tend to regain weight rapidly upon resuming normal eating (Weyer et al., 2000). Even though little is known about the underlying mechanisms, it has been proposed that a fasting-induced reduction in thermogenesis could be caused, at least in part, by substrate cycling inhibition *via* the activation of muscle stearoyl-CoA desaturase 1 (Mainieri et al., 2006). More work is needed to establish whether this enzyme plays a role in reducing re-esterification below baseline in fasting rabbits (Fig. 4A, Fig. 6A).

Shift from re-esterification to oxidation

Fatty acid oxidation is stimulated 2.5-fold during early food deprivation and this response persists when fasting is prolonged (Fig. 4A). Changes in the composition of plasma NEFA suggest that oleate is a preferred fatty acid substrate for oxidation because its relative abundance remains elevated throughout fasting, compensating for a decrease in the percentage of stearate (Table 2). Selectivity for monounsaturated fatty acids like oleate has been demonstrated in many species and at several key steps of lipid metabolism, including mobilization, transport and oxidation (Maillet and Weber, 2006; Weber et al., 2002). The results also show that the percentage of linoleate stays elevated during fasting. However, potential reasons for selecting this particular fatty acid as a preferred fuel for oxidation are presently unclear. Together, increased fatty acid oxidation, decreased TAG/FA cycling (Fig. 4A) and metabolic suppression (Fig. 1B) cause a progressive change in the relative allocation of fatty acids supplied by lipolysis. As energy reserves diminish, the relative fate of fatty acids gradually shifts towards increased oxidation (from 12% to 33%) and decreased re-esterification (from 88% to 67%) (Fig. 4B). New Zeland White rabbits are clearly able to survive without food for much longer than a week [see for example the 32 day fasting study of Parrilla (Parrilla, 1978)]. One month of food deprivation would most likely cause a further increase in relative oxidation and a greater decrease in relative re-esterification than reported here over a 6 day fast (Fig. 4B).

CONCLUSIONS

This study shows that the classic activation of lipolysis caused by short-term fasting is abolished when food deprivation is prolonged. The initial increase in lipolytic rate allows the continued allocation of over 80% of all available fatty acids to re-esterification, even though lipid oxidation has doubled. When energy stores decline, such high fluxes of TAG/FA cycling may become impossible to sustain. Therefore, rabbits may decrease their rates of primary and secondary re-esterification to reduce the cost of substrate cycling. After 6 days without food, the TAG/FA cycle accounts for only 3% of total energy expenditure, even though metabolic rate is now

strongly suppressed. Throughout prolonged fasting, these responses gradually shift fatty acid metabolism towards increased oxidation and reduced re-esterification. Long-term survival is achieved by pushing fuel selection towards the fatty acid dominance of energy metabolism and by slowing substrate cycles to assist metabolic suppression. Interestingly, TAG/FA cycling still remains active after prolonged fasting, suggesting that re-esterification is a crucial mechanism that cannot be stopped without harmful consequences.

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