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Effect of metabolizable protein intake on rates of plasma leucine turnover and protein synthesis in heifers

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SUMMARY

An isotope dilution method using [1-^{13}C]leucine (Leu) infusion together with open-circuit calorimetry was applied to determine the effect of metabolizable protein (MP) intake on rates of plasma Leu turnover and whole body protein synthesis (WBPS) in six heifers. WBPS rate was estimated from rate of plasma Leu turnover and Leu oxidation to carbon dioxide. The experiment consisted of three levels of MP intake and was conducted in a two 3×3 Latin square designs of three 21-day periods. The experimental diet consisted of mixed hay, maize and soybean meal. Dietary MP intake of each dietary treatment was 4·3, 4·5 and 4·9 g/kg $BW^{0.75}$/day by changing maize and soybean meal weights. Metabolizable energy (ME) intake was similar for all dietary treatments. When plasma α-[1-^{13}C]keto-isocaproic acid enrichments were used as markers indicating intracellular Leu enrichments, plasma Leu turnover rate (LeuTR) increased ($P=0{\cdot}012$) and WBPS tended to increase ($P=0{\cdot}091$) as MP intake increased. In contrast, plasma LeuTR and WBPS were not influenced if plasma [1-^{13}C]Leu was taken to indicate intracellular Leu enrichments. Total and plasma Leu oxidation rates did not change but intracellular Leu oxidation increased ($P=0{\cdot}044$) with increasing MP intake. In heifers, it is suggested that rates of plasma Leu turnover and WBPS are influenced by dietary MP intake, independent of ME intake, although the change in MP intake was relatively small.

INTRODUCTION

Amino acids, supplied by absorption from digestive tracts and by degradation from tissue protein, are utilized via a variety of metabolic routes. Leucine (Leu) is, however, either used for protein synthesis or oxidized to carbon dioxide (CO_2) via α-keto-isocaproic acid (α-KIC) (Cobelli *et al*. 1991). Therefore, the isotope dilution method of labelled Leu is widely applied for estimation of whole body protein synthesis (WBPS).

Dietary intake influenced plasma Leu turnover rate (LeuTR) and WBPS in growing cows (Hammond *et al*. 1987; Dawson *et al*. 1998; Lapierre *et al*. 1999). Sano *et al*. (2004) studied the effect of dietary protein intake on WBPS and whole body protein degradation using the [1-^{13}C]Leu method and N balance test in sheep and reported that high protein intake had little effect on plasma LeuTR and WBPS, and enhanced protein deposition with reduced whole body protein degradation rather than increased WBPS. Supply of metabolizable protein (MP), consisting of digestible microbial true protein and digestible undegraded feed protein (AFRC 1993), influenced absorption of essential amino acids, plasma LeuTR and WBPS in lactating cows (Lapierre *et al*. 2002; Raggio *et al*. 2004). Therefore, it was hypothesized that WBPS was affected by dietary MP intake even when metabolizable energy (ME) intake was constant. However, in heifers, less information is available about the effect of MP intake in isoenergetic diets on plasma LeuTR and WBPS. In the present experiment, therefore, plasma LeuTR and WBPS were determined by the [1-^{13}C]Leu method in heifers fed isoenergetic diets estimated to contain three levels of MP. Moreover, plasma and intracellular Leu oxidation rates were separately calculated from both enrichments of plasma [1-^{13}C]Leu and α-[1-^{13}C]KIC as described by Wolfe *et al*. (1982).

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Table 1. *Composition of the dietary treatments*

	Treatment		
	Lo-MP	Me-MP	Hi-MP
Mixed hay (g/kg $BW^{0.75}$/day)	36·7	36·7	36·7
Maize (g/kg $BW^{0.75}$/day)	17·0	12·1	5·2
Soybean meal (g/kg $BW^{0.75}$/day)	1·3	6·3	14·0
CP (g/kg $BW^{0.75}$/day)	6·0	7·8	10·5
MP* (g/kg $BW^{0.75}$/day)	4·3	4·5	4·9
ME* (kJ/kg $BW^{0.75}$/day)	590	588	596

* Assumed from AFRC (1993).

MATERIALS AND METHODS

Animals and diets

Six non-pregnant, non-lactating Holstein heifers, aged 18 ± 2 months and weighing 521 ± 37 kg were used. They were assigned to a two 3×3 Latin square design for 21 days each. The experimental diet consisted of mixed hay of Kentucky bluegrass and Reed canarygrass (3:7; ME 9·2 MJ/kg, 111 g crude protein (CP)/kg air dry matter), maize (ME 13·8 MJ/kg, 82 g CP/kg air dry matter) and soybean meal (ME 13·3 MJ/kg, 427 g CP/kg air dry matter). The dietary treatments consisted of three levels of MP intake – low MP (Lo-MP), medium MP (Me-MP) and high MP (Hi-MP), respectively, as estimated by the Agricultural and Food Research Council (AFRC 1993) and the experimental diets were prepared with changing maize and soybean meal weights (Table 1). ME intake was similar for all treatments (AFRC 1993). CP intake increased with increasing MP intake. The heifers were housed in a stanchion stall in the dairy barn and were fed the mixed hay at 08.30 and 15.30 h and maize and soybean meal at 13.30 and 16.30 h. They were allowed to move outside the house and to rest in a clay field with partial shade after the morning feed. Water was available *ad libitum*. Two catheters for infusion and blood sampling were inserted into both jugular veins on the 20th day and were filled with a sterile solution of 38 g trisodium citrate/l.

Experimental procedures

Combined experiments using open-circuit calorimetry and the isotope dilution procedure for determination of plasma Leu and protein metabolism were carried out in the stanchion stall on the 21st day of each dietary treatment. On the experimental day, the heifers were fitted with a clear head chamber (approximately 1 m^3) for collecting gaseous samples throughout the samplings and were fed the morning diet but were not fed during the [1-^{13}C]Leu infusion. The heifers could freely access an automated waterer in the head chamber. At 09.00 h, 10 μmol/kg $BW^{0.75}$ of [1-^{13}C]Leu (L-leucine-1-^{13}C, 99 atom % ^{13}C; Isotec Inc., A. Matheson, USA) and 3 μmol/kg $BW^{0.75}$ of [^{13}C]sodium bicarbonate (sodium bicarbonate-^{13}C, 99·2 atom % ^{13}C; Isotec Inc., A Matheson, USA) dissolved in saline solution (9 g sodium chloride/l) was injected into the infusion catheter as a priming dose. [1-^{13}C]Leucine was then continuously infused by a multichannel peristaltic pump (AC-2120, Atto Co. Ltd, Japan) at a rate of 10 μmol/kg $BW^{0.75}$/h through the same catheter for 8 h. Blood samples (10 ml) were taken from the sampling catheter immediately before (preinfusion background) and at 30-min intervals during the last 2 h of [1-^{13}C]Leu infusion. Samples were transferred into centrifuge tubes containing heparin sodium and were in ice until centrifugation. Blood samples were centrifuged at 1500 ***g*** for 20 min at 4 °C, and the plasma was stored at −25 °C until analyses. For determination of gaseous metabolism, oxygen (O_2) consumption and CO_2 production of the heifer were analysed by an open-circuit system throughout the experiment. An aliquot of the exhaled gas was collected into 4 ml of 1 N NaOH to determine $^{13}CO_2$ enrichments for 30 min before (preinfusion background) and at 6, 6·5, 7 and 7·5 h of [1-^{13}C]Leu infusion and the NaOH solution was stored at −25 °C until analysis of $^{13}CO_2$ enrichments. After completing determination of gaseous metabolism, the catheters were removed and the heifer was fed the diet. The management and experiment corresponded to the guidelines established by the Animal Care Committee of Iwate University. The experiment was performed without noticeable stress to the heifers.

Analyses

Derivatization of plasma amino acids and α-keto acids was performed by the procedures of Rocchiccioli *et al.* (1981) and Calder & Smith (1988) with slight modifications as described previously (Sano *et al.* 2004). The isotope abundance of plasma [1-^{13}C]Leu and α-[1-^{13}C]KIC derivatives was determined by gas chromatography mass spectrometry (QP-2010, Shimadzu, Japan) with selected ion monitoring. N-leucine and α-ketovaleric acid were used as external standards for determination of plasma Leu and α-KIC concentrations, respectively. To determine the isotope abundance of $^{13}CO_2$, 1 ml of the NaOH solution fixed with exhaled CO_2 was taken in a 3 ml vial which was capped with rubber. After the vial was

vacuumed, the NaOH solution was acidified by injecting 2 ml of 6 N H_2SO_4 using a syringe, and a part of liberated CO_2 was taken by the microsyringe and $^{13}CO_2$ enrichments of liberated CO_2 were determined by gas chromatography/combustion/isotope ratio mass spectrometry (DELTA^{plus}, Thermo Electron Corp., USA). Oxygen consumption and CO_2 production were continuously determined from inlet and outlet air with a respiratory gas analysis system (Metabolic Monitor, Coast Electronics, UK). Concentrations of plasma free amino acids, ammonia and urea at the preinfusion period were determined with an automated amino acid analyser (JLC-500/V, JEOL, Japan). Plasma insulin was determined by a radioimmunoassay kit (IRI 'Eiken', Eiken Chemical Co. Ltd, Japan). The intra- and inter-assay coefficient of variance accounted for were 6 and 9 %, respectively.

Calculations

Mean values with standard errors of the means (S.E.M.) are given. Rates of plasma LeuTR, total Leu oxidation (LeuOX), plasma Leu oxidation (LeuOXpl), intracellular Leu oxidation (LeuOXin) and fractional Leu oxidation (FLeuOX) were calculated using the equations by Goodenough *et al.* (1982) and Krishnamurti & Janssens (1988).

$$\text{LeuTR} = I \times [(1/E) - 1]$$

where I is the infusion rate of [1-^{13}C]Leu and E is the plasma isotope enrichment of [1-^{13}C]Leu or α-[1-^{13}C]KIC during the steady-state conditions.

$$\text{LeuOX} = E_{CO_2}/E_{KIC}/0{\cdot}81 \times V_{CO_2}$$
$$\text{LeuOXpl} = E_{CO_2}/E_{LEU}/0{\cdot}81 \times V_{CO_2}$$
$$\text{LeuOXin} = \text{LeuOX} - \text{LeuOXpl}$$
$$\text{FLeuOX} = \text{LeuOX}/\text{LeuTR}$$

where E_{KIC} and E_{LEU} are the plasma isotope enrichments of α-[1-^{13}C]KIC and [1-^{13}C]Leu, respectively, E_{CO_2} is the isotope enrichment of exhaled $^{13}CO_2$ and V_{CO_2} is the CO_2 production rate. The recovery fraction of the exhaled CO_2 to CO_2 produced in the animal body was assumed to be 0·81 (Allsop *et al.* 1978; Wolfe *et al.* 1982). WBPS was calculated from the following equation.

$$\text{WBPS} = (\text{LeuTR} - \text{LeuOX})/\text{Leu concentration in carcass protein}$$

Leucine concentration in carcass protein was assumed to be 60 g/kg (Lobley *et al.* 1980).

Heat production at 5.5 and 8 h after the initiation of the isotope dilution method was calculated from O_2 consumption and CO_2 production according to the Brouwer's equation (Brouwer 1965) excluding the correction for urinary N and methane production as described by Young *et al.* (1975).

Table 2. *Effect of MP intake on plasma amino acid, ammonia, urea and insulin concentrations at the preinfusion period in heifers*

	Treatment				
(μmol/l)	Lo-MP	Me-MP	Hi-MP	S.E.M.*	*P*-value
No. of heifers	6	6	6		
Arg	134	132	149	6·6	0·282
His	77	77	80	2·5	0·702
Ile	107	114	120	3·9	0·287
Leu	142	152	159	4·0	0·178
Lys	94	95	122	10·3	0·343
Met	25	28	32	1·4	0·086
Phe	53	54	53	1·9	0·955
Thr	84	84	86	4·6	0·795
Val	243	266	275	6·2	<0·01
Ala	234	225	236	9·2	0·371
Asp	14	12	17	1·3	0·390
Glu	100	94	128	6·5	0·034
Gly	335	324	315	10·9	0·759
Pro	79	112	132	9·9	0·068
Ser	106	105	111	5·2	0·951
Asn	32	29	34	1·9	0·507
Gln	376	367	330	12·3	0·271
Tyr	49	47	52	2·2	0·196
Ammonia	182	174	222	12·2	0·240
Urea (mmol/l)	4·4	5·8	6·9	0·37	0·015
Insulin (μU/ml)	12·7	9·9	9·9	0·73	0·066

* S.E.M. = standard errors of the means.

Statistical model

Data were analysed with the MIXED procedure of the SAS (1996). The fixed effects in the model were period and diet. The random effect was heifers. Results were considered significant at the $P<0{\cdot}05$ level. The tendency was defined as $0{\cdot}05 \leqslant P<0{\cdot}10$. If the effect of diet was significant, the Tukey–Kramer adjustment was used and the significance was $P<0{\cdot}05$.

RESULTS

Plasma valine and glutamic acid concentrations at preinfusion increased ($P<0{\cdot}05$) as MP intake increased, but other amino acids were not influenced by MP intake (Table 2). During the preinfusion period, plasma urea concentrations increased ($P=0{\cdot}015$) but plasma ammonia concentrations remained unchanged ($P=0{\cdot}240$) with increasing MP intake. Plasma insulin concentrations tended to decrease ($P=0{\cdot}066$) with increasing MP intake.

Concentrations of plasma Leu and α-KIC and enrichments of plasma [1-^{13}C]Leu and α-[1-^{13}C]KIC and

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Table 3. *Effect of MP intake on plasma concentrations of Leu and α-KIC, rates of Leu turnover, Leu oxidation, WBPS and heat production in heifers*

	Treatment*				
	Lo-MP	Me-MP	Hi-MP	S.E.M.†	*P*-value
No. of heifers	6	6	6	–	
Leu					
Concentration (μmol/l)	144	147	152	7·8	0·470
Turnover rate (μmol/kg $BW^{0·75}$/h)	347	357	388	13·4	0·465
α-KIC					
Concentration (μmol/l)	65	67	63	4·4	0·795
Turnover rate (μmol/kg $BW^{0·75}$/h)	480	511	566	17·9	0·012
Leu oxidation (μmol/kg $BW^{0·75}$/h)					
Total	99	117	140	7·8	0·167
plasma	72	83	97	5·9	0·534
intracellular	27	34	43	3·0	0·044
Fractional Leu oxidation rate	0·20	0·23	0·25	0·011	0·448
Protein (g/kg $BW^{0·75}$/day)					
Intake	6·0	7·8	10·7	0·54	<0·01
Synthesis (Leu)	14·4	14·3	15·2	0·49	0·654
Synthesis (α-KIC)	19·9	20·6	22·3	0·69	0·091
Heat production‡ (kJ/kg $BW^{0·75}$/h)	27·8	29·0	29·5	0·63	0·410
Respiratory quotient‡	0·80	0·80	0·86	0·014	0·175

* Mean values from 6 to 8 h of [1-^{13}C]Leu infusion.
† S.E.M. = standard errors of the means.
‡ Mean values at 5·5 and 8 h of [1-^{13}C]Leu infusion.

exhaled $^{13}CO_2$ were virtually constant during the last 2 h for all of the isotope dilution method (data not shown). The % variance accounted for isotope enrichments during the period were 8·1, 7·8 and 12·6, respectively. Concentrations of plasma Leu and α-KIC did not change with MP intake (Table 3). The enrichments obtained from plasma α-[1-^{13}C]KIC (0·022 ± 0·0008) were lower than those from plasma [1-^{13}C]Leu (0·031 ± 0·0009), and the overall ratio was 0·71 ± 0·018. Therefore, plasma LeuTR calculated from plasma α-[1-^{13}C]KIC enrichments was higher than that from plasma [1-^{13}C]Leu enrichments. Plasma LeuTR increased ($P = 0·012$) with increasing MP intake and was greater ($P < 0·05$) for the Hi-MP diet than for the Lo-MP diet, when plasma α-[1-^{13}C]KIC enrichments were used. However, plasma LeuTR was not influenced by MP intake, when plasma [1-^{13}C]Leu enrichments were used. Total Leu oxidation rate and LeuOXpl did not change ($P = 0·167$ and $P = 0·534$, respectively) but LeuOXin increased ($P = 0·044$) with increasing MP intake and was greater ($P < 0·05$) for the Hi-MP diet than for the Lo-MP diet. Fractional Leu oxidation rate did not change ($P = 0·448$) with MP intake.

WBPS tended to increase ($P = 0·091$) with MP intake, when plasma α-[1-^{13}C]KIC enrichments were used for a precursor. However, WBPS was not influenced ($P = 0·654$) by MP intake, when plasma [1-^{13}C]Leu enrichments were used. Heat production and respiratory quotient were also not influenced ($P = 0·410$ and $P = 0·175$, respectively) by MP intake, but respiratory quotient for Hi-MP diet was numerically higher than the Lo-MP and Me-MP diets.

DISCUSSION

Of the isotopic tracer methods for whole body protein metabolism proposed, the isotope dilution method of [1-^{13}C]Leu with open-circuit calorimetry is widely applied to cows and sheep as well as humans (Lobley 1992), because Leu is either utilized for protein synthesis or oxidized to CO_2 via α-KIC (Cobelli *et al.* 1991). Lapierre *et al.* (1999) studied the effect of feed intake level on protein metabolism using the

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[1-^{13}C]Leu method in growing beef steers and determined both plasma [1-^{13}C]Leu and α-[1-^{13}C]KIC enrichments for estimation of whole body LeuTR, but only plasma α-[1-^{13}C]KIC enrichments were used for WBPS calculation. In another study, Lapierre *et al.* (2002) conducted the same experimental procedure in lactating cows, determined both enrichments of plasma [1-^{13}C]Leu and α-[1-^{13}C]KIC as precursors, and reported that LeuTR was 13–22 % higher plasma α-[1-^{13}C]KIC enrichments than plasma [1-^{13}C]Leu enrichments used for calculation. Matthews *et al.* (1980) suggested that plasma α-[1-^{13}C]KIC enrichments should be used as an indicator of intracellular Leu metabolism. Therefore, the [1-^{13}C]Leu method was applied to heifers, either precursor for calculation of LeuTR and WBPS was determined, and the results obtained from enrichments of plasma α-[1-^{13}C]KIC were discussed.

For application of the isotope dilution method, there are some arguments that the isotope infused influences nutrient utilization in the body (Anthony *et al.* 2000). In the present experiment, the infusion rate of [1-^{13}C]Leu employed was similar to that used in growing beef steers (Lapierre *et al.* 1999) and was lower than that in lactating cows (Lapierre *et al.* 2002). The pooled isotope enrichments of [1-^{13}C]Leu and α-[1-^{13}C]KIC were similar to those reported in growing steers (Lapierre *et al.* 1999). Tessari *et al.* (1985) reported that in humans infusion of stable isotope up to approximately 0·10 of the Leu carbon flux did not have a significant effect on Leu metabolism. Because the infusion rate of [1-^{13}C]Leu was approximately 0·02–0·03 of plasma LeuTR, the isotope infused would not influence plasma LeuTR. Plasma LeuTR was comparable with those reported in growing cows (Hammond *et al.* 1987; Dawson *et al.* 1998; Lapierre *et al.* 1999).

Lobley *et al.* (1987) studied the effect of progressive reduction in food intake on WBPS with the [1-^{14}C]Leu method in finishing beef steers and found that food intake influenced overall protein metabolism. Dawson *et al.* (1998) studied plasma LeuTR in crossbred young steers fed six intake levels, and observed that plasma LeuTR increased with increased dietary intake. It was expected that both dietary energy and N intake influenced WBPS, because the rate of microbial protein synthesis given adequate N intake is believed to be proportional to energy intake (Nolan 1993; Obara *et al.* 1994). Fujita *et al.* (2006) applied a [$^{2}H_{5}$]phenylalanine model to determine the effect of non-protein energy intake on protein metabolism in goats and reported that WBPS was enhanced with increasing dietary energy intake even when dietary CP intake was constant, suggesting that microbial protein, a part of MP, influences WBPS. The present experiment demonstrated that, in heifers, plasma LeuTR increased and WBPS tended to increase with increased MP intake, when enrichments of plasma α-[1-^{13}C]KIC were used. Lapierre *et al.* (2002) reported that in lactating cows producing 16 kg milk/day increased MP supply (1654 and 1930 g/day) enhanced Leu absorption and LeuTR, even though N intake was similar. Although the increment of MP intake in the present experiment (1:1·05:1·14) was slightly less than that (1:1·17) in lactating cows (Lapierre *et al.* 2002) and ME intake was similar between diets, a significant increase in plasma LeuTR and a trend to increase in WBPS were observed. El-Kadi *et al.* (2006) also found that MP intake, increased from 75 to 180 g/day by intraduodenal infusion of casein, enhanced whole body appearance and net absorption of Leu.

Plasma valine, glutamic acid and urea concentrations increased ($P<0·05$) with increasing MP intake, but plasma ammonia concentrations remained unchanged. Raggio *et al.* (2004) studied the effect of MP supply (1922–2517 g/day) on splanchnic flux of amino acids in lactating cows and found that ammonia absorption and hepatic ureagenesis increased but urea recycling decreased with increasing MP supply, resulting in unchanged plasma ammonia concentration and increased plasma urea concentration. Similar changes would occur in the present experiment. Raggio *et al.* (2004) also observed that, in lactating cows, milk production and arterial plasma concentrations of isoleucine, Leu, lysine and valine essential amino acids increased with increasing MP supply, whereas the ratio of the essential amino acids in milk protein relative to portal absorption showed trends to reduce. Because the MP requirement in lactating cows is considerably greater than for growing heifers (AFRC 1993), nutritional and physiological status may be involved in the different trends of plasma amino acid concentrations. The non-significant changes in plasma insulin concentrations with increased MP intake accorded with the result of sheep (Sano *et al.* 2004). In this regard, Sano & Terashima (2001) reported that in sheep, tissue responsiveness and sensitivity to insulin tended to be enhanced with increased dietary CP intake. Tesseraud *et al.* (1993) studied the effect of insulin infusion on Leu metabolism under euglycaemic and eukalemic clamps in lactating and dry goats, and reported that insulin accelerated net Leu balance mainly due to reduced protein degradation even though total Leu flux and non-oxidative Leu disposal was unchanged. Therefore, it may be possible that insulin has less influence on WBPS.

Heat production was comparable with those reported in calves and growing steers (Lobley *et al.* 1987; Chwalibog *et al.* 1996; Derno *et al.* 2005) and was little influenced by MP intake of isoenergetic diets in the present experiment. The higher (but not significant) respiratory quotient for the Hi-MP diet might partly be related to protein oxidation, even though the contribution as energy source was limited

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(Lobley *et al.* 1980). Lapierre *et al.* (1999) reported that in growing beef steers, FLeuOX increased with increasing intake level (9, 17 and 20 % for low, medium and high intake, respectively). Lapierre *et al.* (2002) determined the effect of the supply of MP on whole body Leu kinetics and WBPS in late-lactation dairy cows, and reported that FLeuOX was 21·2 and 16·0 % for the high and middle MP supply, respectively. In the present experiment, FLeuOX was similar to those reported previously but did not increase significantly with increased MP intake. This may partly be a consequence of the small differences in MP intake between diets. It seemed likely that, in lactating cows, more amino acids were utilized as an energy source with increased MP supply (Lapierre *et al.* 2002).

In the present experiment, LeuOX was divided into LeuOXpl and LeuOXin as reported by Wolfe *et al.* (1982) and Goodenough *et al.* (1982) who determined LeuOXpl and LeuOXin separately during exercise and cold exposure, respectively, using both enrichments of plasma [1-^{13}C]Leu and α-[1-^{13}C]KIC in humans. Exercise enhanced LeuOX, LeuOXpl and LeuOXin, whereas cold exposure enhanced LeuOX and LeuOXpl but not LeuOXin. The ratio of LeuOXin to LeuOX in heifers was comparable with that reported in humans (Goodenough *et al.* 1982; Wolfe *et al.* 1982) and LeuOXin increased ($P = 0{\cdot}044$) with increased MP intake, whereas LeuOX and LeuOXpl increased only numerically. These results suggest that the component of LeuOX that occurs in the cell of origin from protein catabolism was enhanced with increased MP intake.

It is suggested that, in heifers, plasma LeuTR and WBPS are influenced by MP intake, even when the increase in MP is relatively modest and ME intake is constant.

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