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Effect of starvation and refeeding on amino acid uptake by mammary gland of the lactating rat

Role of ketone bodies

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1. Arteriovenous differences of amino acids across the mammary glands of lactating rats are diminished when the rats are starved for 24h. 2. When 24h-starved rats were refed for 2½h, the arteriovenous differences of amino acids returned to values similar to those found in well-fed rats. 3. In order to find a possible explanation for these rapid changes, we tested the effect of ketone bodies on amino acid uptake by the gland. At 5min after injection of acetoacetate to fed rats, when the total concentration of ketone bodies in blood was similar to that found in starvation, the uptake of amino acids by the mammary gland was similar to that found after starvation, i.e. lower than in fed rats. However, 30min after administration of acetoacetate, when the arterial concentration of ketone bodies had returned to values similar to those in fed rats, the arteriovenous differences of amino acids were similar to those found in fed rats. 4. We conclude that the changes in blood ketone bodies may be responsible, at least in part, for the changes in amino acid uptake that occur in starvation and in the starvation–refeeding transition.

The uptake of amino acids by mammary gland is tightly controlled. Indeed, we have shown that it is affected by hormonal factors such as prolactin (Viña *et al.*, 1981*c*) or by oestrogens and progesterone (Viña *et al.*, 1981*a*) as well as by local factors (Viña *et al.*, 1981*d*).

The role of nutritional status, particularly starvation and refeeding, on carbohydrate and lipid metabolism has been thoroughly studied (Hawkins & Williamson, 1972; Robinson *et al.*, 1978; Baxter *et al.*, 1978; Munday & Williamson, 1981). However, little was known about the role of the starvation–refeeding transition on amino acid uptake by mammary gland. Here we show that starvation for 24h significantly decreases amino acid uptake by the gland and that this is reversed by short-term (2½h) refeeding. Infusion of acetoacetate to fed rats, to give arterial blood concentrations of ketone bodies similar to those found in starved rats, results in decreases in amino acid uptake similar to those found in starved rats, thus showing that ketone bodies may be very important in the control of amino acid uptake by mammary gland.

Experimental

Animals

Rats at peak lactation were fed *ad libitum* on a standard diet for rats and mice from Prasa, Vara de Quart, Valencia, Spain. All experiments were started between 10:00 and 11:00h. Rats were maintained on a 12h-light/12h-dark cycle, the light period being 08:00–20:00h. In all cases the rats had seven to ten pups. The rats were anaesthetized with Nembutal [50mg/kg body wt. in saline (0.9% NaCl)] and kept with their litters for about 3min after onset of anaesthesia.

Studies in vivo

Samples of blood from rats (well-fed, starved for 24h, or refed for 2½h after 24h starvation) were collected from the aorta and from the pudic–epigastric vein as described by Hawkins & Williamson (1972). The blood samples were deproteinized and used for amino acid analysis.

To study the effect of ketone bodies on amino acid uptake we proceeded as follows. After anaesthesia, a sample of venous blood was collected from the left pudic–epigastric vein into a heparinized syringe with a 25-gauge needle. The inguinal mammary gland of the same side was quickly dissected out and rapidly cut free and clamped between tongs cooled in liquid N_2 (Wollenberger *et al.*, 1960). Then acetoacetate (0.5ml of a 1M solution) or physiological saline (0.5ml) was injected into the femoral vein of the same side. After 5 or 30min a sample of venous blood was collected from the right pudic–epigastric vein, and a piece of inguinal mammary gland of this

side was freeze-clamped. A sample of arterial blood was then immediately collected from the aorta. This experimental procedure is convenient because it permits the measuring of the arteriovenous differences before and after administration of ketone bodies in the same animal. However, it has some shortcomings, particularly that it is very stressful to the animal. In order to test if the surgical procedure used could affect the arteriovenous differences of amino acids across the gland, we performed experiments in which the same experimental procedure was used, except that physiological saline was injected instead of a solution of acetoacetate, and we found that the arteriovenous differences of amino acids were not different from those of control rats (results not shown), thus proving that the effects observed were due to acetoacetate itself and not to the experimental procedure used.

Blood samples were added to 2ml of 6% (w/v) $HClO_4$, and the mixtures were centrifuged in a Sorvall GLC-1 centrifuge at 3000rev./min for 10min to remove protein. The supernatant was neutralized with 20% (w/v) KOH, and the $KClO_4$ precipitate was removed by centrifugation (3000rev./min). This final supernatant was decanted off and used for determination of metabolites. The freeze-clamped tissue was powdered in liquid N_2 with a pestle and mortar, and a portion of the frozen powder (about 1g) was extracted with 4vol. (v/w) of 6% $HClO_4$ by homogenization with a motor-driven Teflon homogenizer. The extract was centrifuged to remove protein and the final supernatant was neutralized with 20% KOH. The $KClO_4$ precipitate was removed by centrifugation. This final supernatant was decanted off and used for assays.

Determination of metabolites

The following metabolites were determined by enzymic methods: glucose (Slein, 1963); L-lactate (Gutmann & Wahlefeld, 1974); pyruvate (Czok & Lamprecht, 1974); acetoacetate and D-3-hydroxybutyrate (Williamson *et al.*, 1962).

Blood samples for amino acid analysis were treated as described by Viña *et al.* (1981*b*).

Results

Effect of starvation and refeeding on arteriovenous differences of amino acids across the mammary gland

Table 1 shows that the arteriovenous differences of amino acids across the mammary gland of lactating rats are greatly diminished when compared with controls. Although the arterial concentrations of amino acids in 24h-starved rats are lower than in controls, the ratio arteriovenous differences/arterial concentration of amino acids is diminished, thus showing that the decreased uptake

Table 1. *Effects of starvation and refeeding on arteriovenous differences of amino acids across the mammary gland of the rat*

For details see the text. Values are mean ± S.D. for the numbers of experiments in parentheses. Arteriovenous differences that are statistically different from controls are expressed: $*P < 0.005$, $**P < 0.0005$.

	Controls (6)		Starved for 24h (7)		Refed for 2½h after 24h starvation (5)	
Amino acid	Arterial concn. (μM)	Arteriovenous differences (nmol/ml)	Arterial concn. (μM)	Arteriovenous differences (nmol/ml)	Arterial concn. (μM)	Arteriovenous differences (nmol/ml)
L-Aspartic acid	36 ± 5	10 ± 2	31 ± 5	4 ± 1**	33 ± 5	8 ± 2
L-Threonine	432 ± 98	108 ± 14	309 ± 75	16 ± 16**	338 ± 59	84 ± 10
L-Serine	340 ± 81	82 ± 27	219 ± 14	16 ± 22**	287 ± 36	59 ± 18
L-Asparagine	60 ± 11	18 ± 6	50 ± 5	7 ± 4*	65 ± 4	21 ± 3
L-Glutamic acid	213 ± 16	29 ± 5	153 ± 16	−3 ± 15**	201 ± 17	34 ± 6
L-Glutamine	589 ± 35	138 ± 30	449 ± 50	39 ± 15**	648 ± 64	164 ± 25
L-Proline	266 ± 49	47 ± 14	162 ± 12	17 ± 9*	237 ± 10	38 ± 11
Glycine	301 ± 71	58 ± 15	203 ± 16	−3 ± 27**	254 ± 25	44 ± 6
L-Alanine	546 ± 102	131 ± 15	388 ± 73	−9 ± 24**	665 ± 82	151 ± 33
L-Valine	170 ± 37	69 ± 24	137 ± 26	21 ± 8**	185 ± 5	49 ± 11
L-Cystine	137 ± 15	41 ± 8	100 ± 13	12 ± 8**	115 ± 9	38 ± 5
L-Methionine	115 ± 11	40 ± 8	72 ± 8	14 ± 8**	93 ± 12	32 ± 3
L-Isoleucine	113 ± 12	45 ± 10	63 ± 12	11 ± 6**	81 ± 8	29 ± 7
L-Leucine	191 ± 20	71 ± 20	129 ± 18	20 ± 5**	173 ± 9	74 ± 3
L-Tyrosine	123 ± 21	36 ± 11	81 ± 12	10 ± 9*	110 ± 4	20 ± 6
L-Phenylalanine	41 ± 9	15 ± 5	35 ± 4	2 ± 3**	49 ± 3	18 ± 2
L-Lysine	139 ± 11	29 ± 5	90 ± 9	8 ± 2**	121 ± 1	16 ± 3*
L-Histidine	203 ± 11	35 ± 9	143 ± 20	8 ± 5**	180 ± 8	28 ± 4
L-Arginine	63 ± 2	20 ± 4	46 ± 5	4 ± 3**	62 ± 5	17 ± 2

of amino acids is not a result of a diminished arterial concentration. However, when 24h-starved rats were refed ($2\frac{1}{2}$h), both the arterial concentration of amino acids and the arteriovenous differences across the mammary gland returned to values similar to the controls.

Effect of ketone bodies on amino acid uptake by mammary gland

The short-term changes in amino acid uptake by mammary gland observed in the starved–refed transition indicated that metabolic changes that occur in this situation might be responsible for the changes in amino acid uptake by the gland.

Ketone bodies were considered a possible signal for amino acid metabolism. Indeed, their role as a signal to control glucose metabolism by the gland was proposed by Robinson & Williamson (1977). Thus we tested the effect of administration of acetoacetate as described in the Experimental section.

Table 2 shows that 5min after acetoacetate injection the concentrations of acetoacetate and 3-hydroxybutyrate were significantly higher than in controls, resulting in a total concentration of ketone bodies of 1.5mM, a concentration found in normal human beings after starvation (Williamson & Hems, 1970), but somewhat higher than that found by Hawkins & Williamson (1972) in the 16h-starved lactating rat. However, 30min after acetoaceate injection the concentration of ketone bodies returned to control values. The concentrations of glucose, lactate and pyruvate were not affected by injection of acetoacetate (Table 2).

Table 2. *Concentrations of metabolites in blood of fed lactating rats after injection of acetoacetate or saline*
The results are mean values ± S.D., expressed as μmol/ml of whole blood. The numbers of rats are shown in parentheses. Arterial samples were collected after injection as described in the Experimental section. Values that are statistically different after acetoacetate injection from those after saline injection are shown by: $*P < 0.005$.

	Metabolite concn. (μmol/ml of whole blood)				
Injection	Glucose	Pyruvate	Lactate	Acetoacetate	3-Hydroxybutyrate
Saline, 5min (6)	4.08 ± 0.50	0.10 ± 0.03	1.71 ± 0.46	0.08 ± 0.02	0.13 ± 0.02
Acetoacetate, 5min (10)	4.01 ± 0.77	0.09 ± 0.05	2.10 ± 0.06	0.70 ± 0.28*	0.76 ± 0.17*
Saline, 30min (4)	3.80 ± 0.40	0.05 ± 0.02	1.78 ± 0.04	0.11 ± 0.04	0.20 ± 0.10
Acetoacetate, 30min (8)	3.94 ± 0.46	0.04 ± 0.01	1.40 ± 0.42	0.07 ± 0.02	0.16 ± 0.06

Table 3. *Effect of acetoacetate injection on arteriovenous differences of amino acids through lactating mammary gland*
For details see the text. Results are mean ± S.D., for the numbers of rats shown in parentheses. Arteriovenous differences that are statistically different (by Student's *t* test) before and after acetoacetate injection are shown by: $*P < 0.05$; $**P < 0.005$.

	Acetoacetate injection (5min) (7)			Acetoacetate injection (30min) (8)		
	Arterial concn.	Arteriovenous differences (nmol/ml)		Arterial concn.	Arteriovenous differences (nmol/ml)	
Amino acid	(μM)	Before injection	After injection	(μM)	Before injection	After injection
L-Aspartic acid	35 ± 5	9 ± 2	3 ± 0**	35 ± 3	10 ± 2	10 ± 3
L-Threonine	332 ± 75	56 ± 22	13 ± 8**	314 ± 68	79 ± 17	79 ± 30
L-Serine	249 ± 26	57 ± 14	12 ± 6**	260 ± 36	61 ± 14	54 ± 23
L-Asparagine	65 ± 8	20 ± 2	8 ± 4**	66 ± 8	23 ± 5	25 ± 5
L-Glutamic acid	196 ± 19	37 ± 11	16 ± 14*	197 ± 12	37 ± 8	37 ± 13
L-Glutamine	607 ± 33	167 ± 28	52 ± 56**	615 ± 41	171 ± 17	158 ± 20
L-Proline	235 ± 24	41 ± 15	14 ± 8**	251 ± 10	38 ± 5	39 ± 13
Glycine	244 ± 25	41 ± 15	18 ± 17*	241 ± 25	42 ± 20	37 ± 13
L-Alanine	517 ± 62	155 ± 29	17 ± 16**	538 ± 24	161 ± 11	153 ± 18
L-Valine	153 ± 13	42 ± 12	14 ± 7**	166 ± 20	48 ± 3	53 ± 11
L-Cystine	113 ± 10	43 ± 3	11 ± 7**	123 ± 13	37 ± 11	36 ± 10
L-Methionine	85 ± 4	33 ± 5	4 ± 4**	90 ± 7	40 ± 4	33 ± 10
L-Isoleucine	86 ± 12	30 ± 10	10 ± 10**	88 ± 11	37 ± 5	30 ± 3
L-Leucine	180 ± 12	70 ± 5	16 ± 6**	189 ± 20	74 ± 6	73 ± 6
L-Tyrosine	108 ± 9	28 ± 6	10 ± 5**	114 ± 8	29 ± 8	27 ± 8
L-Phenylalanine	46 ± 4	14 ± 3	4 ± 2**	51 ± 4	17 ± 3	14 ± 2
L-Lysine	126 ± 10	21 ± 5	5 ± 3**	123 ± 5	21 ± 7	18 ± 5
L-Histidine	177 ± 10	29 ± 6	7 ± 1**	186 ± 20	30 ± 9	23 ± 9
L-Arginine	57 ± 6	15 ± 1	3 ± 1**	61 ± 4	17 ± 4	16 ± 4

Table 3 shows that 5min after acetoacetate injection, i.e. when the total concentration of ketone bodies was 1.5mM, the arteriovenous differences of amino acids were significantly lower than in controls. However, 30min after acetoacetate injection, i.e. when the arterial concentration of ketone bodies had returned to control values, the arteriovenous differences of amino acids were not significantly different from controls. This effect of ketone bodies may explain, at least in part, the decrease of amino acid uptake by the gland induced by starvation.

Sodium acetoacetate was used in these experiments. Fery & Balasse (1980) found that infusion of $NaHCO_3$ for 3h had similar effects to sodium acetoacetate on alanine metabolism by human skeletal muscle, thus showing the importance of the cation on the effect of sodium acetoacetate in muscle amino acid metabolism. To test if the observed effect was due to the amount of Na^+ injected, we injected 0.5ml of 1M-$NaHCO_3$ (i.e. the same molar amount as that used with sodium acetoacetate) and found that the arteriovenous differences of amino acids were similar to those of controls (results not shown), thus showing that the observed effects are due to acetoacetate itself and not to the amount of Na^+ administered.

Discussion

The arteriovenous differences of amino acids are greatly decreased by starvation for 24h. However, they return to control values after the animals are refed for 2½h. We have observed that γ-glutamyl transpeptidase is involved in amino acid uptake by mammary gland (Viña *et al.*, 1981*b*) and that changes in the activity of this enzyme induced by prolactin (Viña *et al.*, 1981*c*), by weaning (Viña *et al.*, 1981*d*) or by oestrogens and progesterone (Viña *et al.*, 1981*a*) are always followed by parallel changes in amino acid uptake. Thus we tried to find if γ-glutamyl transpeptidase activity in mammary gland was affected by starvation or by ketone bodies, but found that neither affected this activity in mammary gland, thus showing that the observed effects are independent of the γ-glutamyl cycle (Meister, 1973).

Robinson & Williamson (1977) suggested that changes in redox state of the tissue owing to ketone-body utilization might affect mammary-gland metabolism. Indeed, changes in [NAD^+]/[NADH] ratio may inactivate pyruvate dehydrogenase (Denton *et al.*, 1975). We found that 5min after acetoacetate injection the [lactate]/[pyruvate] ratio in freeze-clamped mammary gland decreased from 24.4 to 11.3, which suggests that the cytoplasmic [NAD^+]/[NADH] ratio is increased. At 30min after acetoacetate injection the [lactate]/[pyruvate] ratio in freeze-clamped gland was similar to that in the gland before the injection. Thus the changes in the cytoplasmic [NAD^+]/[NADH] ratio, which are related to the increase in blood ketone bodies, may be, at least in part, responsible for the decreased uptake of amino acids by the gland. Changes in redox state of the cytosolic [NAD^+]/[NADH] couple have been shown to affect protein synthesis and proteolysis in skeletal muscle. Indeed, increases in the [NAD^+]/[NADH] ratio promote increases in the rate of proteolysis (Tischler, 1980) and decreases in the rate of protein synthesis (Hedden & Buse, 1982) in muscle. In the present paper we show that increases in [NAD^+]/[NADH] ratio promoted by the administration of ketone bodies decreases amino acid uptake by the gland. These changes in amino acid uptake are not followed by parallel changes in amino acid concentration in the mammary gland. Thus the concentration of amino acids in mammary glands freeze-clamped 5min after injection of acetoacetate was similar to that found in controls (results not shown) in spite of the amino acid uptake being dramatically decreased (Table 3). This shows that the changes in amino acid uptake are probably due to metabolic changes induced by ketone bodies and probably mediated by changes in the [NAD^+]/[NADH] ratio. Since the arteriovenous differences of all the amino acids are affected, it is very likely that the changes are related to protein synthesis and/or proteolysis.

The uptake of substrates by an organ depends on blood flow as well as on arteriovenous differences. We have measured arteriovenous differences. However, under the circumstances studied, the blood flow is either unaltered or must change in the same direction as the arteriovenous differences. Indeed, mammary-gland blood flow may decrease in the starved rat, as occurs in the goat (Annison *et al.*, 1968). Thus the changes in net amino acid uptake by the gland will be greater than the arteriovenous differences indicate. Arteriovenous differences have been used to estimate the uptake of carbohydrate and lipid metabolites by the gland (Hawkins & Williamson, 1972).

In the present paper we show that amino acid uptake by mammary gland is decreased by 24h starvation. This effect is completely reversed by short-term (2½h) refeeding. These rapid changes in amino acid uptake may be caused by changes in blood concentration of ketone bodies, because administration of acetoacetate to fed rats promotes an inhibition of amino acid uptake. The fact that an increase in blood ketone bodies brings about a decrease in uptake of glucose (Robinson & Williamson, 1977) as well as in amino acid uptake by mammary gland provides a common mechanism to explain the inhibition of lactation during starvation.

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