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Responses to homeostatic signals in ractopamine-treated pigs

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The β-agonist ractopamine (RAC) promotes protein deposition with little effect on fat deposition in the pig. To assess whether the lack of effect on fat deposition was due to changes in response to homeostatic signals, eight crossbred gilts (73 kg body weight (BW)) with venous catheters were used to examine plasma metabolite and hormone concentrations before and after intravenous injections of insulin and the β_2-agonist fenoterol during dietary RAC (0 or 20 mg/kg) treatment. Pigs received intravenous challenges of insulin (1 μg/kg BW) on days 3, 9 and 23 and fenoterol (2 μg/kg BW) on days 4, 10 and 24 of treatment. RAC was then withdrawn from the diet and insulin and fenoterol challenges were repeated 6 and 7 d later respectively. Blood samples for the determination of metabolite and hormone concentrations were taken at −30, −20, −10, −1, 2·5, 5, 10, 20, 30, 45, 60 and 120 min relative to the challenges. Dietary RAC decreased basal plasma insulin concentrations but had no effect on plasma glucose or non-esterified fatty acids (NEFA). Hypoglycaemic responses to insulin were not affected by RAC while the anti-lipolytic effects of insulin tended to be augmented. Dietary RAC decreased the lipolytic response to fenoterol, this being evident after 4 d treatment. Hypoglycaemic response to fenoterol was not changed whereas the hyperinsulinaemic response to fenoterol was attenuated by dietary RAC. Previous treatment with RAC did not influence basal hormone and metabolite concentrations or responses to homeostatic signals during the withdrawal period. While these results suggest little change in glucose metabolism, the de-sensitization of adipose tissue β-adrenergic receptors is consistent with the observations that dietary RAC has little effect on the rate of fat deposition in the growing pig.

β-Agonist: Non-esterified fatty acids: Glucose: Pig

Previous studies have demonstrated that the β-agonist ractopamine (RAC) is a stimulator of protein deposition in growing pigs (Mitchell *et al.* 1990, 1991; Dunshea *et al.* 1993*a*, *b*, *c*). However, effects on lipid deposition have been more equivocal. While body-fat content and backfat depths have been consistently decreased by RAC and other β-agonists, in the few studies where the actual rate of lipid deposition has been measured by slaughter balance the effects are far from consistent. For example, we have failed to observe any decrease in the rate of lipid deposition (i.e. g/d) in pigs fed on a wide range of energy and protein intakes (Dunshea *et al.* 1993*a*, *b*, *c*). Mitchell *et al.* (1990) found that RAC decreased lipid deposition in their low-select line (selected for lean growth when fed on diet containing 120 g crude protein (CP)/kg) of barrows when they were fed on a ration containing 120 g CP/kg but not when fed on a 240 g CP/kg diet. Lipid deposition was not altered by RAC in their high-select (selected for lean growth when fed on a diet containing 240 g CP/kg) line of pigs irrespective of dietary protein content. RAC decreased lipid deposition in barrows when fed *ad lib.* but not when fed restrictively (Mitchell *et al.* 1991). Therefore, it appears that under some circumstances RAC and possibly other β-agonists can decrease lipid deposition in pigs but that this effect is relatively small and variable in magnitude. The aim of the present experiment was to determine whether responses to homeostatic signals such as β-adrenergic stimulation or insulin are altered during dietary RAC treatment and thus contribute to the relative lack of effect on fat deposition observed

during RAC treatment of pigs. The β_2-agonist fenoterol was chosen as the model β-agonist since porcine adipose tissue contains predominantly β_2-adrenergic receptors (Coutinho *et al.* 1990).

MATERIALS AND METHODS

Animals and surgery

Eight crossbred (Large White × Landrace) gilts (initial body weight (BW) 73 kg) were maintained in individual pens throughout the study. Animals were housed under a 14 h light–8 h dark cycle with lights being turned on at 07.00 hours. Pigs were catheterized at least 7 d before commencement of the study. Muscle relaxation was induced with an intramuscular injection of azoperone (Stresnil, 40 mg/ml; Boehringer, NSW, Australia) after which anaesthesia was induced and maintained with halothane (Fluothane; ICI, Victoria, Australia). A silastic catheter (Sil-Med Corporation, Taunton, MA, USA; 0·16 mm i.d., 0·32 mm o.d.) was inserted 150 mm into the anterior *vena cava* via the cephalic vein (Takken & Williams, 1981). The catheter was exteriorized in the region of the interscapular space on the back of the animals and stored in a cloth pocket glued to the back. After catheterization pigs were given a 4 d course of broad spectrum antibiotic (Terramycin/LA, oxytetracycline 200 mg/ml; Pfizer, NSW, Australia) and exit wounds were treated with antibiotic powder (Terramycin, oxytetracycline, 20 mg/g; Pfizer). Catheters were flushed daily with physiological saline containing K_2EDTA (12·5 g/l).

Experimental procedures

For 4 d before initiation of the dietary treatments pigs were offered 500 g of a nutrient-dense diet (Table 1) every 4 h. Commencing at 08.00 hours on day 1 pigs were offered their ration containing either 0 or 20 mg ractopamine.HCl/kg for 24 d. From day 25 until day 30 all pigs were fed on the diet containing 0 mg RAC/kg. Pigs received an intravenous challenge of insulin (Actrapid MC; Novo Industri, Copenhagen, Denmark; 1 μg/kg BW) on days 3, 9 and 23 to determine plasma metabolite and hormone responses to insulin. Challenges of the β_2-agonist fenoterol (Sigma Chemical Co., St Louis, MO, USA; 2 μg/kg BW) were administered on days 4, 10 and 24 to determine plasma metabolite and hormone responses to β-adrenergic stimulation. Insulin and fenoterol challenges were again administered on days 30 and 31 (i.e. 6 and 7 d after withdrawal of RAC) respectively. Blood samples (8 ml) were taken at −30, −20, −10, −1, 2·5, 5, 10, 20, 30, 45, 60 and 120 min relative to challenges and placed in heparinized tubes (60 U/ml) on ice. After low-speed centrifugation, plasma was harvested and stored at −70° until analysed for glucose, non-esterified fatty acids (NEFA) and insulin.

Plasma analyses

Plasma glucose was analysed using an enzymic kit assay based on linked glucose oxidase (*EC* 1.1.3.4)–peroxidase (*EC* 1.11.1.7) reactions (Sigma Chemical Co.; Cat. no. 510). Plasma NEFA were analysed using an enzymic kit assay based on linked acyl-CoA synthetase (*EC* 6.2.1.3)–acyl-CoA oxidase (*EC* 1.3.3.6)–peroxidase reactions (Boehringer Mannheim, Germany; Cat. no. 1082 914) modified to conduct additional assays by a five-fold dilution of all reagents with 0·025 M-potassium phosphate buffer (pH 7·8). Plasma insulin was analysed using a commercial kit (Amersham, Bucks.) using recombinant human insulin as a standard. Analyses for each metabolite or hormone for each challenge day included plasma samples obtained from four pigs (two from each treatment). All analyses were performed in duplicate with pools included in each assay. Inter- and intra-assay coefficients of variation were 2·7 and 1·1%, 11·4 and 2·7% and 9·0 and 5·3% for glucose, NEFA and insulin respectively.

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Table 1. *Composition of the experimental diet**

Ingredient	g/kg
Wheat	774·6
Soya-bean meal	116·1
Meat-and-bone meal	37·65
Blood meal	30·0
Fat blend	20·0
Limestone	8·56
Dicalcium phosphate	5·72
L-Lysine.HCl	2·5
DL-Methionine	0·80
Vitamin and mineral premix†	2·0
Salt	2·0

* Diet formulated to contain 183 g crude protein/kg, 14·5 MJ DE/kg and 10·8 g lysine/kg.
† Provided the following nutrients (mg/kg air-dry diet): retinol 6·4, cholecalciferol 0·083, α-tocopherol 22, menadione 0·60, riboflavin 3·3, nicotinic acid 16·5, pantothenic acid 5·5, pyridoxine 1·1, biotin 0·56, choline 1100, cyanocobalamin 0·017, Fe 88, Zn 55, Mn 22, Cu 6·6, I 0·22, Se 0·1.

Table 2. *Basal non-esterified fatty acid (NEFA), glucose and insulin concentrations and metabolic responses to insulin or fenoterol in pigs given dietary ractopamine (RAC)**

	Day of dietary treatment							Significance‡		
	3/4		9/10		23/24			RAC	DAY	RAC × DAY
Ractopamine.HCl (mg/kg)...	0	20	0	20	0	20	SED†	(df 6)	(df 12)	(df 12)
Basal plasma concentrations§										
NEFA (μmol/l)	43·5	47·5	44·7	33·9	43·7	37·4	5·5	NS	NS	0·062
Glucose (mmol/l)	5·12	5·16	4·92	4·86	4·61	4·56	0·24	NS	< 0·001	NS
Insulin (mU/l)	41·4	28·0	34·0	25·3	31·5	25·8	4·1	0·002	NS	NS
Response to insulin‖										
NEFA (μmol min/l)	−114	−304	−22	−120	−86	−74	101	0·075	NS	NS
Glucose (mmol min/l)	−81	−102	−73	−67	−64	−85	9	NS	0·025	NS
Response to fenoterol¶										
NEFA (μmol min/l)	2314	1071	2206	554	2538	855	194	< 0·001	NS	NS
Glucose (mmol min/l)	11	14	29	13	23	22	7	NS	NS	NS
Insulin (mU min/l)	340	219	621	252	567	232	183	0·023	NS	NS

* Pigs were fed on diets containing either 0 or 20 mg ractopamine.HCl/kg for 24 d. For further details see p. 810.
† Standard error of the difference for treatment means.
‡ NS, $P > 0·10$.
§ Values are the means of samples taken at −30, −20, −10 and −1 min before insulin and fenoterol injections (plasma NEFA and glucose) or before fenoterol injection (plasma insulin).
‖ Area under the curve (corrected for basal) after intravenous injection of insulin (1 μg/kg body weight) on days 3, 9 and 23 of dietary ractopamine treatment.
¶ Area under the curve (corrected for basal) after intravenous injection of fenoterol (2 μg/kg body weight) on days 4, 10 and 24 of dietary ractopamine treatment.

Calculations and statistics

Responses to homeostatic signals were expressed as area under the metabolite or hormone *v.* time curve for the next 1 h or until circulating metabolite or hormone concentration returned to basal, whichever came first. Basal concentrations were determined as the

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Table 3. *Basal non-esterified fatty acid (NEFA), glucose and insulin concentrations and metabolic responses to insulin or fenoterol in pigs previously given ractopamine**

	Ractopamine.HCl (mg/kg)			
	0	20	SED†	Significance‡ (df 6)
Basal plasma concentrations§				
NEFA (μmol/l)	35·2	35·3	4·7	NS
Glucose (mmol/l)	4·61	4·41	0·11	NS
Insulin (mU/l)	28·8	21·2	3·78	NS
Response to insulin‖				
NEFA (μmol min/l)	−12	−52	60	NS
Glucose (mmol min/l)	−71	−91	7·7	NS
Response to fenoterol¶				
NEFA (μmol min/l)	2464	2333	712	NS
Glucose (mmol min/l)	22	18	6·0	NS
Insulin (mU min/l)	322	185	139	NS

* Pigs were fed on diets containing either 0 or 20 mg ractopamine.HCl/kg for 24 d. Insulin and fenoterol challenges were conducted 6 and 7 d after withdrawal of ractopamine respectively. For further details, see p. 810.

† Standard error of the difference for treatment means.

‡ NS, $P > 0{\cdot}10$.

§ Data are the means of samples taken at −30, −20, −10 and −1 min before insulin and fenoterol injections (plasma NEFA and glucose) or before fenoterol injection (plasma insulin).

‖ Area under the curve (corrected for basal) after intravenous injection of insulin (1 μg/kg body weight) on day 6 after withdrawal of dietary ractopamine treatment.

¶ Area under the curve (corrected for basal) after intravenous injection of fenoterol (2 μg/kg body weight) on day 7 after withdrawal of dietary ractopamine treatment.

average of the samples taken at −30, −20, −10 and −1 min relative to the challenge. Statistical analyses were performed on basal concentrations averaged for days 3 and 4 (d 3/4), days 9 and 10 (d 9/10), days 23 and 24 (d 23/24) and days 30 and 31 (withdrawal). Data from the treatment period were analysed using an analysis of variance suitable for a split-plot design with treatment as the main plot and time as the subplot. Error terms used for F statistics were pig within RAC for evaluating significance of RAC treatment and (pig within RAC) × day for evaluating day effects and interactions. Data from the withdrawal period were compared using Student's t test.

RESULTS

Animal performance

The performance of these pigs has been reported elsewhere (Dunshea & King, 1994). Briefly, average daily gain between days 1 and 24 was increased by RAC (754 (SE 30) *v.* 1030 (SE 41) g/d for control *v.* RAC treatment). For the week following withdrawal of RAC there was no difference in average daily gain (674 (SE 43) *v.* 736 (SE 38) g/d).

Basal metabolite and hormone concentrations

Basal plasma NEFA concentrations were not significantly affected by dietary RAC or time (Table 2). While plasma glucose was not affected by dietary RAC, it did decrease with time (Table 2). Basal plasma insulin was significantly reduced by dietary RAC. During the withdrawal period there were no significant differences in basal plasma NEFA, glucose or insulin between the control animals and those that had previously received RAC (Table 3).

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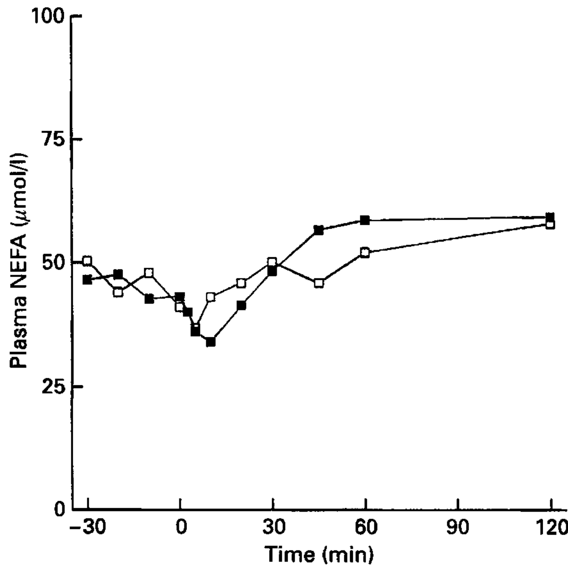

Fig. 1. Plasma non-esterified fatty acid (NEFA) response after intravenous challenge with insulin (1 μg/kg body weight) in gilts given 0 (□, *n* 4) or 20 (■, *n* 4) mg ractopamine/kg diet. Values are means for challenges conducted on days 3, 9 and 23 of treatment.

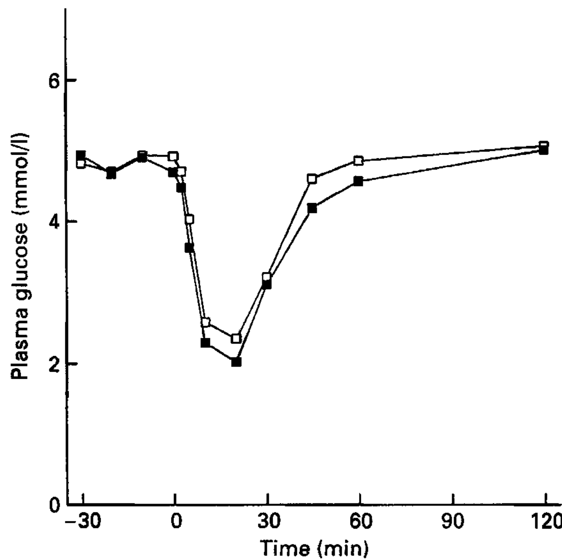

Fig. 2. Plasma glucose response after intravenous challenge with insulin (1 μg/kg body weight) in gilts given 0 (□, *n* 4) or 20 (■, *n* 4) mg ractopamine/kg diet. Values are means for challenges conducted on days 3, 9 and 23 of treatment.

Responses to insulin

Insulin was anti-lipolytic as evidenced by a small decrease in plasma NEFA after insulin challenge (Fig. 1), with the plasma NEFA response tending to be greater ($P = 0{\cdot}075$) in RAC-treated gilts (Table 2). The decrease in plasma NEFA after insulin injection during the withdrawal period was not different between treatment groups (Table 3). Insulin

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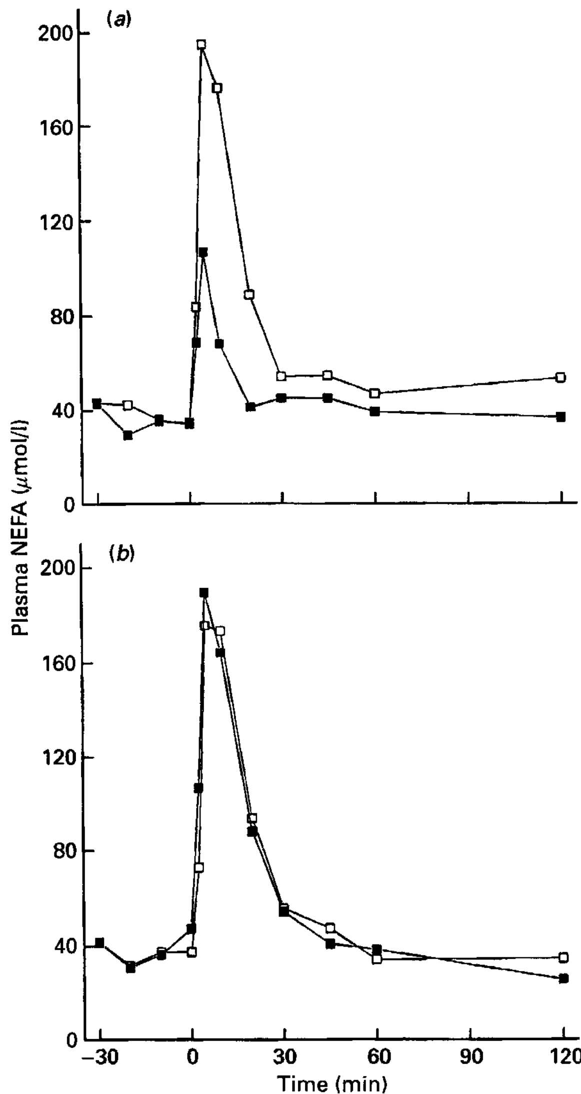

Fig. 3. Plasma non-esterified fatty acid (NEFA) response after intravenous challenge with fenoterol (2 μg/kg body weight) in gilts given 0 (□, *n* 4) or 20 (■, *n* 4) mg ractopamine/kg diet. Values are means for challenges conducted (a) on days 4, 10 and 24 of treatment or (b) after 7 d of withdrawal of RAC.

injection caused acute hypoglycaemia in all pigs although there was no difference between treatments (Fig. 2, Table 2). The hypoglycaemic response to insulin decreased with time, possibly because basal blood glucose concentrations also decreased. Prior RAC treatment had no effect on the hypoglycaemic effects of insulin during the withdrawal period (Table 3).

Responses to fenoterol

Intravenous challenge with fenoterol stimulated lipolysis as evidenced by an acute increase in plasma NEFA (Fig. 3). However, the lipolytic response was markedly reduced in RAC-treated gilts (Fig. 3(a), Table 2). Lipolytic responses returned to control values by 7 d after

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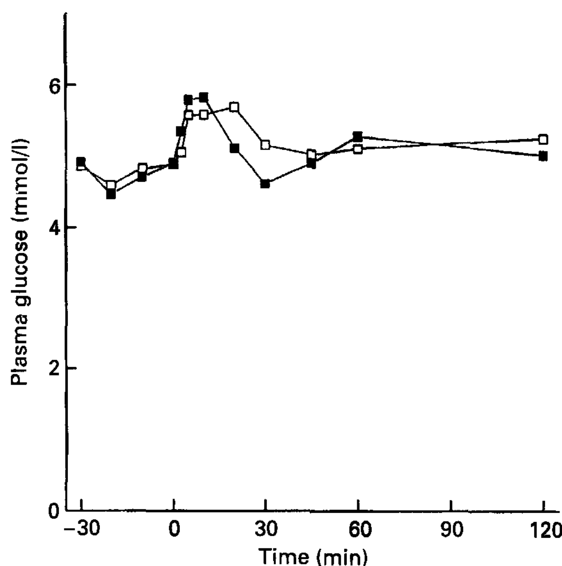

Fig. 4. Plasma glucose response after intravenous challenge with fenoterol (2 μg/kg body weight) in gilts given 0 (□, *n* 4) or 20 (■, *n* 4) mg ractopamine/kg diet. Values are means for challenges conducted on days 4, 10 and 24 of treatment.

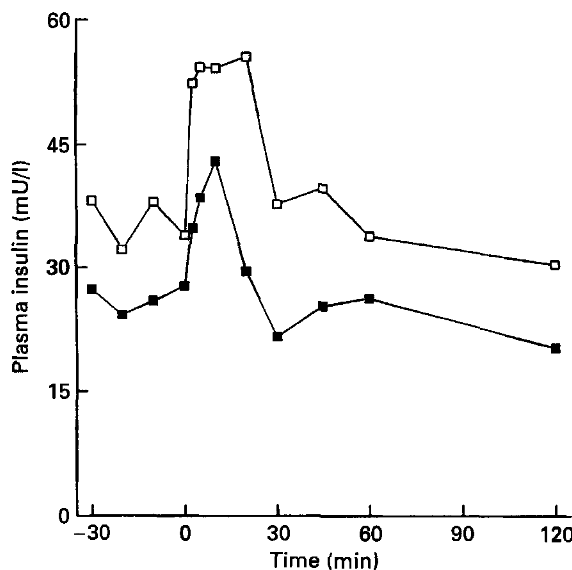

Fig. 5. Plasma insulin response after intravenous challenge with fenoterol (2 μg/kg body weight) in gilts given 0 (□, *n* 4) or 20 (■, *n* 4) mg ractopamine/kg diet. Values are means for challenges conducted on days 4, 10 and 24 of treatment.

withdrawal of RAC (Figure 3(b), Table 3). Intravenous fenoterol caused mild hyperglycaemia but there was no difference between treatment groups either during dietary RAC treatment (Fig. 4, Table 2) or during the withdrawal period (Table 3). Plasma insulin also increased after fenoterol injection with this response being diminished during dietary

RAC treatment (Fig. 5, Table 2). The fenoterol-induced hyperinsulinaemia during the withdrawal period was not affected by prior RAC treatment.

DISCUSSION

Lipid metabolism

There was no significant effect of RAC on basal plasma NEFA concentrations suggesting that RAC did not stimulate lipid mobilization or lipolysis, or if it had, that these effects had disappeared by day 3. Interestingly, there was the suggestion of an interaction between day and RAC ($P < 0.062$), such that basal plasma NEFA decreased with time on treatment. This may occur if there is de-sensitization of adipose-tissue adrenergic response to both endogenous and exogenous stimulation. This certainly appeared to be the case with the dramatic decrease in the lipolytic response to the β_2-agonist fenoterol. Thus, after only 4 d dietary RAC treatment, while basal NEFA concentrations were not different, the lipolytic response to fenoterol was reduced to only 44% of that of the control gilts. On days 9 and 24 the NEFA responses to fenoterol challenge were only 25 and 34% that of the control pigs respectively. Response returned to control values 7 d after withdrawal. These results suggest that RAC treatment leads to de-sensitization of adipose-tissue β-adrenergic receptors. This apparent de-sensitization is evident as early as 4 d after RAC treatment and is maintained for at least 3 weeks. Withdrawal of RAC results in an equally rapid return to normal adrenergic lipolytic response.

In support of our findings *in vivo*, Spurlock *et al.* (1993) recently reported that feeding RAC for 24 d reduced adipose-tissue adrenergic receptor density by 50% (as assessed with isoproterenol) with differences being detectable as early as 1 d after feeding RAC. In contrast, Adeola *et al.* (1992) reported that the NEFA response to the ($\beta_1 + \beta_2$)-agonist isoproterenol (1 μg/kg BW) was greater in RAC-treated pigs receiving a diet containing 170 g CP/kg but unchanged when the basal diet contained 130 g CP/kg. However, while basal *in vitro* lipolysis was not affected by dietary RAC, isoproterenol-stimulated lipolysis was reduced (Adeola *et al.* 1992). The reason for the different responses to the fenoterol and isoproterenol challenges is unknown but may be that the challenges were conducted on different parts of the dose–response curve. Porcine adipose tissue is more sensitive to isoproterenol than fenoterol by at least an order of magnitude whereas maximum responsiveness is not different (Mersmann, 1987). Therefore, RAC may be decreasing adipose tissue sensitivity (seen with fenoterol) but not maximum responsiveness (observed with isoproterenol) to β-adrenergic stimulation. In this context, decreased β-adrenergic sensitivity with unchanged responsiveness was evident in acute co-incubations of porcine adipocytes with RAC or clenbuterol and epinephrine (Liu & Mills, 1989). Dietary clenbuterol also decreased *in vitro* adipose-tissue β-adrenergic sensitivity without changing responsiveness (Mills & Orcutt, 1989). It should also be borne in mind that the basal plasma NEFA concentrations in the study of Adeola *et al.* (1992) were at least ten times higher than in the present study and are indicative of fasted and/or stressed pigs. Therefore, it is possible that their pigs had not received feed (including dietary RAC) for some time. Given the rapidity with which de-sensitization of adipose β-adrenergic receptors occurs (Spurlock *et al.* 1993), it is possible that the study by Adeola *et al.* (1992) was conducted after re-sensitization had occurred.

Insulin was anti-lipolytic although the magnitude of the response was relatively small (Fig. 1). In part this was due to the already low basal plasma NEFA concentrations and presumably lipolysis in these pigs. However, the response in the RAC-treated pigs tended to be greater ($P = 0.075$) than in the control pigs. If this is a real effect of RAC then it would ensure that RAC-stimulated lipolysis is kept in check by the augmented anti-lipolytic effect

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of insulin. In this context, basal plasma insulin was decreased by dietary RAC which could in turn be a response to the increased anti-lipolytic effect of insulin.

Glucose metabolism

Despite the lower circulating levels of insulin and possible augmented anti-lipolytic effect of insulin, basal blood glucose concentrations and the hypoglycaemic effect of insulin were unchanged by dietary RAC. Therefore, RAC appears to have little effect on the ability of insulin to stimulate glucose uptake by peripheral tissues *in vivo*. However, β-agonists such as RAC, cimaterol and isoproterenol antagonize the acute lipogenic and anti-lipolytic effects of insulin in porcine adipose tissue (Liu *et al.* 1989; Peterla & Scanes, 1990) possibly through reduced binding to adipocyte insulin receptors. Incubation of porcine adipocytes with RAC and clenbuterol decreased insulin binding at low (physiological) but not high media insulin concentrations (Liu & Mills, 1990). Whether this phenomenon occurs *in vivo* is still unclear. For example, while co-incubation of mouse adipocytes with RAC and clenbuterol also reduced insulin binding, adipocytes from mice treated with these β-agonists *in vivo* actually had higher insulin binding than adipocytes from control mice (Dubrovin *et al.* 1990). Therefore, there appear to be some disparate effects of RAC on acute *in vitro* compared with chronic *in vivo* actions of insulin. However, it should be noted that the chronic *in vivo* effects were consistent with our observations that dietary RAC has little effect on the rate of lipid deposition (Dunshea *et al.* 1993*a*, *b*, *c*).

Fenoterol-induced hyperglycaemia was not affected by dietary RAC, consistent with what is observed when RAC-treated pigs are challenged with isoproterenol (Adeola *et al.* 1992). Hyperglycaemia after adrenergic stimulation is generally attributed to hepatic glycogenolysis and so these results suggest that there is little change in the ability of the pig liver to respond to adrenergic stimulation during RAC treatment. However, in challenge-type studies there are always counter-regulatory mechanisms operating. For example, after adrenergic stimulation there is also a period of hyperinsulinaemia, presumably in response to increased plasma glucose and NEFA. The hyperinsulinaemic response to fenoterol was reduced during RAC treatment, possibly due to the decreased NEFA response, and this may also reduce the hyperglycaemic response to fenoterol.

Conclusions

In conclusion, these results support the hypothesis that the lack of effect of dietary RAC on the rate of lipid deposition in the pig is due to a rapid de-sensitization of adipose-tissue β-adrenergic receptors. Withdrawal of RAC results in an equally rapid return to normal adrenergic lipolytic response. Dietary RAC also appears to have little effect on glucose metabolism although basal insulin concentrations are decreased.

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