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TECHNICAL NOTE

TIME AND DOSE RESPONSE OF BLOOD NON-ESTERIFIED FATTY ACIDS TO ADRENERGIC STIMULATION IN RABBIT DOES

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Abstract: The response in blood non-esterified fatty acids (NEFA) to an adrenergic stimulation was studied in rabbit does. Two separate experiments were carried out to find the best time interval for blood sampling after an adrenergic stimulation and to find the appropriate dose for the challenging stimulus. In the first experiment, the time evolution was studied by injecting 9 animals intravenously with 50 mg of isoproterenol per kg of live weight (LW), blood being collected just before and 5, 10, 15, 20, 30 and 40 minutes after challenge. The highest NEFA responses, calculated as the difference between NEFA concentration in samples taken before and after adrenergic stimulation, were observed at 5 and 10 minutes after challenge (+0.70±0.15 and +0.77±0.15 mmol/L, respectively). In the second experiment, 6 animals were consecutively injected with isoproterenol (0.5, 5 or 50 mg/kg LW) and placebo (NaCl, 0.9%) with an interval of 1 hour; and blood was sampled just before and 7.5 minutes after challenge. The NEFA responses were significantly different from zero only when isoproterenol was injected at the dose of 50 mg/kg LW. Thus, we concluded that, among the tested dose concentrations of isoproterenol, 50 mg/kg LW was the most useful dose for adrenergic stimulation and that the adrenergic response was highest at 5 and 10 minutes after challenge.

Key words: rabbit, adrenergic stimulation, non-esterified fatty acids, dose response, time response.

INTRODUCTION

Mobilisation of body reserves contributes to the energy supply of reproductive animals, especially during periods of high demand (CHILLIARD *et al.*, 1998). This energy is released from fat cells into the blood as non-esterified fatty acids (NEFA)

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under the action of lipase. Therefore there is a strict correlation between the rate of NEFA release from adipose tissue and its blood concentrations. Isoproterenol, a potent non selective β-agonist, is one of the most efficient regulators of the adipose tissue and the evolution of blood NEFA levels in response to an isoproterenol challenge has been found a useful tool to evaluate the lipolytic potential of the fat reserves (CHILLIARD *et al.*, 1998). The NEFA response is a function of time from injection, concentration of isoproterenol and the lipolytic potential in the adipose tissue. Thus, the aim of this experiment was to define the time interval between injection of isoproterenol and sampling of the stimulated value, and to define an appropriate challenging dose in order to use this technique in rabbit does in future experiments.

MATERIALS AND METHODS

Two experiments were carried out: the first studied the time evolution, while the second analysed the dose response. The experiments were performed at the experimental rabbit farm of the Department of Animal Science, Polytechnic University of Valencia, Spain. Multiparous crossbred New Zealand White female rabbits obtained from the cross of the V and A lines of the cited Department were used. All animals were fed a commercial diet *ad libitum*. All animals were weighed before entering the experiment (4500±412 g). Both experiments were performed in the morning hours to prevent the effect of feeding time and other activities in the farm, although as the animals were fed *ad libitum* the effect of feeding time was expected to be scarce.

Experiment 1 (Time evolution)

A total of 10 multiparous does either dry or in late lactation were used. Different physiological states were used as a means to increase the variation of the NEFA response. Animals were fixed in hammocks for ileal sample collection to minimise movements approximately 10 minutes before first challenge, so that animals would have time to become accustomed to the new environment. The animals were kept in these hammocks during the whole experimental period. The ear was shaved and a

catheter was arranged in the central ear artery. One of the animals was used as a basal reference by the injection of a placebo (NaCl 0.9%). Blood was sampled at time zero and immediately thereafter either isoproterenol [I5627, Sigma, 50 µg/kg of live weight (LW)] or placebo were injected by direct puncture in the marginal ear vein. The isoproterenol was diluted using 1 mL of the solution for an animal weighing 4 kg. Blood samples were collected at 5, 10, 15, 20, 30, and 40 minutes after challenge. After each collection, sodium citrate 0.3% was injected into the catheter to prevent blood coagulation inside. After clothing, blood samples centrifuged (1100 *g*, 4 °C, 10 min) and serum was separated and stored at -18°C until assayed. All blood samples were analysed for NEFA.

Experiment 2 (Dose response)

Having settled the time interval between injections of isoproterenol and sampling of the stimulated value, the following step was to investigate the effect of dose on the lipolytic response. Three different doses of isoproterenol (0.5, 5, or 50 µg/kg LW) and a placebo (NaCl 0.9%) were prepared. Six lactating animals were used for this experiment. A balanced Latin square design was used, so all animals were subject to all treatments, but in a different order. The isoproterenol challenge was carried out as described above; animals were challenged with one of the four treatments, and the blood was sampled just before and 7.5 minutes after challenge. The subsequent challenge was performed with a time interval of one hour, when NEFA have returned to basal level according to the results of the first experiment. This procedure was repeated until each animal had undergone all treatments, and animals were kept in their hammocks during the procedure. In this experiment, the tubes used for the blood samples were EDTA prepared and plasma was used instead of serum because the centrifugation could be done immediately after blood sampling and the centrifugation could be done with the tubes wherein the samples were taken, thereby reducing the protocol in the laboratory. The blood samples were centrifuged (3000 *g*, 4 °C, 15 min) and plasma stored at -18ºC until NEFA determination. In cases where either the basal or the stimulated values were not obtained for a dose, this observation was excluded for further analysis. Due to problems with the sampling procedure, the number of observations of placebo and treatment 0.5, 5 or 50 µg isoproterenol/kg LW were 4, 3, 6, and 5, respectively.

NEFA determination

NEFA determination was performed using the acyl-CoA synthetase-acyl CoA oxidase (ACS-ACOD) method as prepared in the Wako C kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Samples were analysed in an autochemical analyser Wako Diagnostics 20R (Toshiba Medial Systems Co., Ltd., Tokyo, Japan). The within-run precision of this technique is 2.7, 1.1, and 1.1% of CV for mean concentrations of 0.33, 0.62 and 0.99 mmol/L, respectively. The linear range of the assay is 0-2.0 mmol/L, thus the analyser automatically diluted and reanalysed the samples when the concentration of the NEFA where above the working range.

Statistical analysis

In the first experiment, the responses were calculated as the difference between the NEFA concentration after adrenergic stimulation and the basal concentration (time 0). A mixed model was used for variance analysis of the response, including time point (six times) and physiological state (dry *vs* lactating) as fixed effect. Random effect of animal was also included. In the second experiment, the responses were calculated as the individual differences between the NEFA concentration after adrenergic stimulation and the basal NEFA concentration for each challenge. A general lineal model including dose (4 levels) as fixed effect and random effect of animal was used. In both experiments, the generalised least square means of the response were tested if different from zero. The variances were calculated using the VCE4 software (GROENEVELD, 1998) and these variances were used for the statistical tests performed with the PEST package (GROENEVELD, 1990).

RESULTS AND DISCUSSION

Time evolution

The blood NEFA concentration increased after an isoproterenol challenge, and the average response values significantly ($P<0.05$) differed from zero at 5, 10 and 15 minutes after challenge (Table 1). The maximum levels were reached at 5 (+0.70±0.15 mmol/L) and 10 (+0.78±0.15 mmol/L) minutes after challenge and

Table 1: Least square means of blood NEFA response (difference between NEFA concentrations after and before stimulation) to an isoproterenol challenge, standard error and contrast between estimated values and zero for six time points after challenge.

Minutes after challenge	NEFA response mmol/L	S.E.	Ho: response=0 *P*-value
5	+0.70	0.15	< 0.001
10	+0.77	0.15	< 0.001
15	+0.49	0.15	0.02
20	+0.17	0.15	0.27
30	+0.04	0.15	0.79
40	+0.11	0.18	0.55

from this moment on a slow decrease of the response was observed. The NEFA concentrations were not significantly different from the basal levels from 20 minutes after challenge. The response at 15 minutes could not be discarded, being no different (P=0.15) from the average response at 5 and 10 minutes. The NEFA response for the control animal did not change during the experimental period (Figure 1), anyway as only one animal was used this results cannot permit to be sure that NEFA do not increase due to sampling stress. REVERTE and RIVAS-CABAÑERO (1996) administered 0.3 µg isoproterenol/kg LW/min during 30 minutes and observed a similar evolution of serum NEFA, as they observed the maximum response immediately after ending the administration and the response decreased by 60% 15 minutes later. Anyway, as the isoproterenol administration protocol was quite different, a comparison should be taken with great care.

Dose response

According to the results of the first experiment, a time span between challenge and sampling of 7.5 minutes was chosen for the dose response experiment. The NEFA responses to the challenge (Table 2) were only significantly (P<0.01) different from zero when injecting 50 µg/kg LW. The stimulated NEFA values for this challenge dose were found to be on average 1.7 times higher than the basal level, which is intermediate among the 1.4 fold increase observed in lactating cows (THEILGAARD *et*

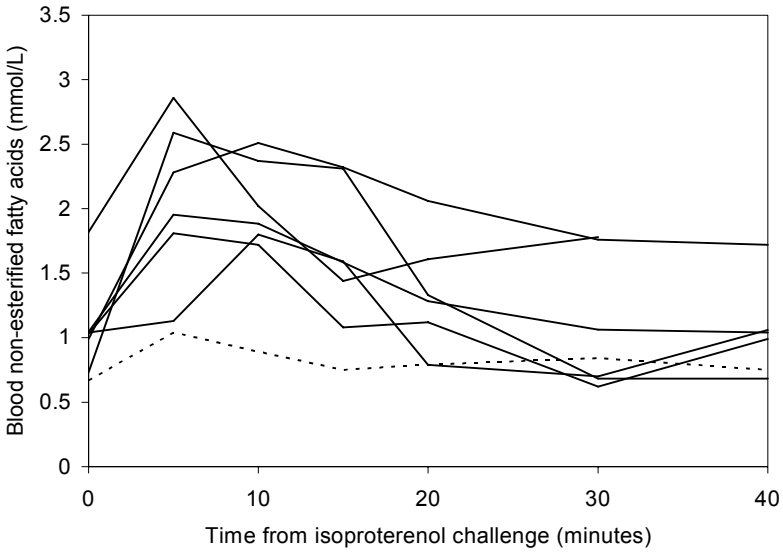

Figure 1: Blood non-esterified fatty acids (NEFA) concentration of 6 rabbit does subjected to isoprotenerol (——) challenge (50 µg/kg LW) and (- -) a control doe injected with placebo (NaCl 0.9%).

al., 2002), the 1.65 fold response in fasting rabbit in a long-term administration of isoproterenol (REVERTE and RIVAS-CABAÑERO, 1996) and the 1.8-2.8 fold increase in rats (UYSAL *et al.*, 2000). The concentration used in this experiment is higher than what has hitherto been reported used for fasted rabbits (a total of 9 µg/kg LW distributed along 30 minutes REVERTE and RIVAS-CABAÑERO, 1996), but only half of what has been used in mice (0.1 mg CL 316.243 (a β-agonist)/kg LW) in a similar experimental protocol where animal were not fasted previously (UYSAL *et al.*, 2000).

Table 2: Least square means of NEFA response (difference between NEFA concentration after and before stimulation), standard error, contrast between estimate and zero shown for four treatments.

Treatment	NEFA response mmol/L	S.E.	Ho: response=0 *P*-value
Placebo	+0.04	0.19	0.83
0.5 µg/kg LW	-0.24	0.22	0.30
5 µg/kg LW	+0.10	0.16	0.54
50 µg/kg LW	+0.54	0.17	0.01

CONCLUSION

In this preliminary work we found that the NEFA responses were highest at 5 and 10 minutes after challenge and were maintained significantly different from zero also after 15 minutes. Among the tested doses, the stimulation with an isoproterenol concentration of 50 μg/kg LW significantly raised the NEFA level. Since the optimal dose is though likely both to be smaller or bigger than 50 μg/kg LW we found effective, the results should be interpreted as useful values for investigating adrenergic response, but not necessary the optimal value. Further, there is still no evidence of if this method is useful for detecting metabolic differences according to physiological state, genetic line and management. Future experiments are needed for clarifying these points.

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