Effects of nutrition on testicular growth in mature Merino rams actively immunized against GnRH

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Nutrition-induced changes in testicular size in Merino rams appear to involve both GnRH-dependent and -independent pathways. This hypothesis was tested by feeding mature Merino rams that had been actively immunized against BSA or GnRH conjugated to BSA a diet that maintained initial body weight or the same diet supplemented daily with 1.5 kg of lupin grain. Blood was sampled every 20 min for 24 h on days 1, 19 and 70 relative to the change in diet. The plasma was used to assess the effects of treatments on changes in LH, FSH and testosterone concentrations. In the group immunized against BSA, FSH increased in lupin-supplemented rams compared with maintenance-fed rams, while LH and testosterone were not affected by diet. In comparison, the concentrations of LH, FSH and testosterone were significantly lower in the group immunized against GnRH than in rams immunized against BSA, but none of these endocrine variables was affected by nutrition. With both immunization treatments, the testes were significantly larger in lupin-supplemented than in maintenance-fed rams. In the group immunized against BSA, this difference was caused by testicular growth in lupin-supplemented rams, whereas in the group immunized against GnRH, lupin supplementation effectively maintained testicular mass, rather than allowed the regression observed in maintenance-fed rams. In conclusion, differences in testicular growth that were induced by dietary treatments in rams immunized against GnRH were not associated with changes in gonadotrophin or testosterone secretion. This supports the hypothesis that part of the effect of nutrition on testicular growth is independent of changes in GnRH secretion. The differences in testicular size observed in control rams were of similar magnitude to those observed in treated rams, but associated with large differences in plasma FSH concentrations, suggesting that this hormone plays an important role in this effect.

Introduction

In mature Merino rams, changes in nutrition can modulate testicular growth (Salaman, 1964; Lindsay et al., 1976, 1984; Oldham et al., 1978; Ritar et al., 1984; Schoeman and Combrink, 1987; Thwaites and Hannan, 1989; Martin et al., 1987, 1994a; Hötzel et al., 1995a) and the production of spermatozoa (Salaman, 1964; Oldham et al., 1978). These responses have also been demonstrated in rams of other breeds (Alkass et al., 1982; Lindsay et al., 1984) but, in the Merino, the responses are powerful enough to override the effects of photoperiod (Masters and Fels, 1984; Martin et al., 1994b, 1994c).

The classical view of regulation of testicular function is that the effects of internal and external stimuli (for example puberty, photoperiod and social interactions) are relayed to the testis by the gonadotrophins, LH and FSH, the secretion of which is determined by changes in GnRH pulse frequency. However, the effect of nutrition appears to be mediated in a different way, because changes in testicular growth are poorly associated with changes in gonadotrophin secretion (Ritar et al., 1984; Martin et al., 1987, 1994a). Recent work has shown that the concentrations of LH and FSH do change shortly after a change in diet, but these responses are transient and values return to basal values after a few weeks while testicular growth continues (Martin et al., 1994a). This finding led to the suggestion that the effects of nutrition on testicular growth in rams are partly independent of changes in GnRH secretion (Martin et al., 1994a). This view was reinforced by studies showing: (i) a high correlation between changes in body weight and in testicular growth (Murray et al., 1990); (ii) a similar dissociation between gonadotrophin patterns and nutrition-induced changes in testicular growth in goat bucks (Walkden-Brown et al., 1994), and (iii) even greater dissociation
between gonadotrophin patterns and nutrition-induced effects on ovulation rate in sows and ewes (Cox et al., 1987; Ritar and Adams, 1988; Scaramuzzi and Campbell, 1990; Downing et al., 1995). The observations in females are supported by increases in ovarian activity, independent of effects on LH or FSH secretion, after metabolites or metabolic hormones are infused directly into the ovary (Cox et al., 1987; Downing and Scaramuzzi, 1991; Downing et al., 1995).

In a recent study this hypothesis was tested by imposing an exogenous regimen of high frequency GnRH pulses on rams, typical of the short-term response they experience on a supra-maintenance diet, and examining the effects on testicular growth in rams fed supra- or sub-maintenance diets (Hötzel et al., 1995a). The infusion of GnRH produced similar plasma profiles for LH, FSH and testosterone in rams fed the two diets, but testicular size was significantly reduced by undernutrition in rams receiving the exogenous GnRH infusion, supporting the hypothesis. The present complementary study further tested the hypothesis, by imposing low concentrations of GnRH by actively immunizing the rams against GnRH. The effect of nutrition on the steroidogenic capacity of the testes of those rams was also determined.

Materials and Methods

Animals and experimental design

The experiment was conducted at the University of Western Australia (latitude 31°56'S), under natural lighting. Photoperiod in this region ranges from 10 h light:14 h dark in winter to 14 h light:10 h dark in summer. At the beginning of the experiment, in mid-November (spring), it was about 13.5 h light:10.5 h dark. During the experiment, the animals were kept in individual pens with food provided once a day and fresh water available ad libitum. The experiment was preceded by an acclimatization period of 2 weeks during which the animals were fed 800 g day⁻¹ of a basal diet consisting of a mixture of oat chaff with 10% lupin grain (Lupinus angustifolius) and 3% (w/w) mineral supplement (Siromin, CSIRO, Australia).

On day −1 of the experiment, 20 mature Merino rams were randomly allocated to treatments in a 2 × 2 factorial experiment with two diets and two types of immunization (n = 5 per group), on the basis of body weight (mean ± SEM: 60 ± 1.9 kg) and scrotal circumference (mean ± SEM: 34.4 ± 0.4 cm). The rams were actively immunized against BSA (BSA-i) or against GnRH conjugated to BSA (GnRH-i). Within each immunization treatment, half of the rams were fed 1 kg of the basal diet described above (maintenance diet) and the other half were fed the same diet supplemented with 1.5 kg lupin grain daily (high diet).

Collection of blood samples

Blood samples were taken every 20 min for 24 h on days −1 and 19 and for 12 h on day 70 (07:00 h to 19:00 h) from polyethylene jugular cannulae fitted the day before. On day 77 testicular responsiveness was tested by an intravenous injection of ovine LH (NIADDK-oLH-25; 200 ng kg⁻¹ body weight), followed by blood sampling for 4 h at 30 min intervals, starting 10 min before the injection of LH. Single blood samples were also taken each week by venepuncture and used to measure GnRH antibody titres and FSH concentrations. Every week, the rams were weighed and scrotal circumference was measured at the widest point with the animals standing and the testes hanging loosely in the scrotum. These procedures were carried out early in the morning before feeding.

Immunization procedure

The immunogen consisted of 1 mg GnRH conjugated to 4 mg BSA by the glutaraldehyde reaction, as described by Caraty et al. (1980). The conjugate was lyophilized for storage and reconstituted in physiological saline before use. The first injection was emulsified in Freund’s complete adjuvant (CSL, Parkville, Victoria, Australia) and consecutive booster injections were emulsified in incomplete Freund’s adjuvant (CSL). A dose of 1 mg antigen in a total volume of 5 ml was injected subcutaneously in at least ten sites on the back of the animals on days −10, 1, 10, 20, 30, 40 and 70 of the experiment. Control animals were injected with 4 mg BSA emulsified in the same adjuvants.

Assessment of GnRH antibody titres

The tracer was (3-[(125)iodotyrosil-5]-GnRH that had been labelled with 125I by the chloramine-T method to specific activity 74 TBq mmol⁻¹. On day 1 of the assay, 100 µl samples that had been serially diluted (1:400, 1:2000 and 1:4000) in phosphate-buffered saline with 0.1% (w/v) gelatin, pH 7.4 (GPB) were incubated at room temperature with 100 µl labelled GnRH and 200 µl GPB. After 9 h, 100 µl donkey anti-sheep serum (raised in our laboratory and diluted 1:20 in GPB) was added and incubated overnight at 4°C. On day 2, 1 ml GPB containing 6% (w/v) polyethylene glycol 6000 was added; the tubes were centrifuged for 30 min at 2000 g; the supernatant was aspirated and the amount of radioactivity in the pellet was measured. Titre was defined as the dilution of plasma that bound 30% of added tracer in the final volume of 400 µl.

Hormone assays

Luteinizing hormone was measured in all plasma samples. Follicle stimulating hormone was measured in the samples taken each week and in every seventh sample during each serial sampling (the mean for the whole period of serial sampling for each ram was used to calculate group means). Testosterone was measured in all the samples taken after the LH challenge and in every second sample taken on day 70.

Plasma concentrations of LH were measured in all samples using the radioimmunoassay described by Martin et al. (1994a). The preparation CNRS-M3 (biopotency 1.8 IU NIH-LH-S1 mg⁻¹) used for reference was kindly supplied by M. Jutisz (Collège de France). The tracer was prepared using NIIDKK-oLH-I-3 donated by the National Institute of Diabetes, Digestive and Kidney Disease (Baltimore, MD). The antiserum R1 was raised in a rabbit in our laboratory and exhibited the following cross-reactions; 100% with NIH-LH-S1, 97% with

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NIH-LH-S20, 18% with NIAMDD-oFSH-RP1, 0.93% with NIH-FSH-S12, 8.2% with oGH and 5% with NIH-TSH-S8. The limit of detection of the standard curve (for all assays) was calculated by subtracting two standard deviations from the mean counts bound in nine replicates of the zero standard. Samples were assayed as duplicate 200 µl aliquots and the limit of detection was 0.25 ng ml⁻¹. Interassay coefficients of variation (mean ± SEM) were 12.1 ± 1.4%, 6.9 ± 1.2% and 12.5 ± 2.2%, for quality controls containing 6.9, 1.4 and 0.6 ng ml⁻¹, respectively. The interassay coefficients of variation were 13.8%, 9.32% and 16.1%, respectively.

Plasma concentrations of FSH were assayed by the method described by Martin et al. (1994a) using a kit kindly supplied by the National Institute of Diabetes, Digestive and Kidney Disease. The kit comprised antiserum oFSH-1, reference preparation oFSH-RP-1 (biopotency 75 IU NIH-FSH-S1 mg⁻¹) and tracer preparation ofFSH-1-1. The samples were run in a single assay as duplicate 200 µl aliquots and the limit of detection was 0.18 ng ml⁻¹. Mean interassay coefficients of variation were 17.9, 16.1 and 7.1 for quality controls containing 6.2, 3.4 and 1.7 ng ml⁻¹, respectively.

Plasma testosterone was measured using a non-extraction radioimmunoassay, as described by Hötz et al. (1995a). The antiserum (Rabbit) 3 was raised in our laboratory against testosterone-3-CMO-HSA. The preparation 4-androstene-17β-ol-3-one (10 µg ml⁻¹; Sigma Chemicals Co., MO) was used for reference and the tracer was 1,2,6,7-[3H]testosterone (Amersham International, Buckinghamshire). Crossreactions were 100% with testosterone, 70% with dihydrotestosterone, 3.7% with androstenedione, and less than 0.05% with progesterone, oestradiol, oestrone and oestriol. The limit of detection of the assay was 0.12 ng ml⁻¹ and the interassay coefficients of variation (mean ± SEM) were 8.7%, 6.8% and 10.6% for quality controls containing 5.9, 3.4 and 0.4 ng ml⁻¹, respectively.

Statistical analysis

Repeated measures analysis of variance was applied to all variables to test for interactions between the effects of diet and immunization. When main effects or interactions were significant (P < 0.05), one-way analysis of variance was applied within periods to test for interactions between the effects of diet and immunization at that time. Fisher’s Protected LSD was used to test for differences between treatments within a period when appropriate. The FSH data were logarithmically transformed because the variance was directly proportional to the mean, but untransformed data are presented.

Results

**GnRH antibody titres**

Rams immunized against GnRH had a low antibody titre throughout the experiment, taking about 9 weeks to reach 1:400, the maximum titre reached. Rams immunized against BSA never displayed antibody titres above the nonspecific binding of the assay (mean 5.8% binding in 1:400).

**Change in body weight and scrotal circumference**

There were significant effects of diet (P < 0.0001) and the time × diet interaction (P < 0.0001) on the change in body weight (Fig. 1a). Immunization did not affect the rate of change in body weight throughout the experiment. Body weight increased in the rams fed the high diet and did not change in rams fed the maintenance diet, so that it differed significantly between the two diets at the end of the experiment (P < 0.001).

There was a significant effect of diet (P < 0.004) and immunization (P < 0.0001) on the change in scrotal circumference (Fig. 1b) but no interactions between the effects of diet and immunization. On day 77, the difference in scrotal circumference between rams fed the high and maintenance diets was similar within each immunization group. The change in scrotal circumference was positive and higher (P < 0.01) in BSA-i rams fed the high diet than in any other group; GnRH-i rams fed the high diet and BSA-i rams fed the maintenance diet maintained
higher

Luteinizing hormone and follicle stimulating hormone

There was a significant interaction between the effects of immunization and time on LH pulse frequency and mean LH pulse amplitude ($P < 0.01$). None of these variables differed among the groups on day $-1$ of the experiment, so the data were pooled for presentation (Fig. 2). On days 19 and 70, mean LH pulse frequency and mean LH pulse amplitude were lower in GnRH-i rams than in BSA-i rams ($P < 0.02$), but did not differ between the diets.

Repeated measures analysis of variance revealed an effect ($P < 0.05$) of the interaction between diet, time and immunization on plasma FSH concentrations (Fig. 3a). The values were higher in BSA-i than in GnRH-i rams on day $-1$ of the experiment ($P < 0.04$), and remained different throughout the observation period. Diet affected mean FSH concentrations in BSA-i but not in GnRH-i rams. In BSA-i rams fed the high diet, FSH concentrations remained fairly constant until day 35, and thereafter increased, to reach 450% of initial values by the end of the experiment (Fig. 3b), by which time they were significantly higher than those in all other groups ($P < 0.03$). In all the other groups, mean FSH concentrations remained relatively unchanged throughout the experiment.

Testosterone

Rams immunized against GnRH had significantly lower plasma testosterone concentrations than BSA-i rams on day 70 ($P < 0.0005$; Fig. 4a). However, when adjusted to mean LH concentrations (calculated as ratio of mean testosterone to mean LH concentrations), there were no differences between treatments (Fig. 4b).

The injection of exogenous oLH on day 77 of the experiment produced a pulse of LH of similar amplitude ($5.5 \pm 0.59$ ng ml $^{-1}$) in all treatment groups. The testosterone response to this challenge, calculated as area under the testosterone curve following the peak in plasma LH, was lower in GnRH-i rams than in BSA-i rams ($P < 0.04$; Fig. 4c). Again, when testosterone was adjusted to mean LH concentrations (Fig. 4d), the groups did not differ from one another.

Discussion

In mature Merino rams, a high plane of nutrition effectively arrests the testicular regression induced by immunization against GnRH. The observation that nutrition can affect testicular size when GnRH is held at low concentrations adds a corollary to our recent study showing that nutrition can affect testicular size when GnRH is held at high concentrations (Hötzel et al., 1995a). These two complementary experiments both support the hypothesis that part of the effect of nutrition on testicular growth in mature rams is independent of changes in GnRH secretion.

The increase in scrotal circumference in control rams fed the high diet was associated with an increase in plasma FSH concentrations. However, in rams immunized against GnRH, diet sustained testicular mass despite low concentrations of LH and FSH, so that testicular size was independent of changes in gonadotrophin secretion. This finding adds to several earlier studies that challenge the view that there is an obligatory association between long-term changes in gonadotrophin concentrations and testicular growth, at least when such growth is induced by changes in nutrition (Ritar et al., 1984; Martin et al., 1987, 1994a; Walkden-Brown et al., 1994). In control rams, the high diet increased testicular size rather than maintained it, so testicular growth is still dependent upon adequate gonadotrophic support. The ultimate effect of diet on testicular growth was of similar magnitude whether gonadotrophin concentrations were normal or lower than normal, suggesting that the GnRH-dependent and GnRH-independent mechanisms have additive effects on testicular mass. This might be because factors that affect testicular function at the intratesticular level have to interact with the gonadotrophins to either alter the
responsiveness of target cells to gonadotrophins or modulate their actions on these cells (Sharpe, 1984).

Testicular growth in control rams fed the high diet was not associated with an increase in LH pulse frequency on days 19 and 70. This finding is consistent with our previous observation that LH pulse frequency is stimulated by an increase in diet but then returns to normal within 3–4 weeks (Martin et al., 1994a), that is, before the first intensive blood sampling in the present study. However, in the present experiment, the high diet led to a large increase in FSH concentrations that was sustained for a long period, as found by Hötzel et al. (1995a), suggesting that FSH plays an important role in the stimulatory effect of nutrition on testicular growth. This observation can be linked to the fact that, in the growing testis, the seminiferous tissue, but not the interstitial tissue, is developing (Hötzel et al., 1995b) and it is consistent with the relatively greater importance of FSH over LH in the control of spermatogenesis in rams (Courot, 1988; Kilgour et al., 1993, 1994).

While the lack of effect of diet on LH pulse frequency is consistent with previous findings (Ritar et al., 1984; Martin et al., 1987, 1994a), the sustained changes in FSH concentrations in this and the previous study (Hötzel et al., 1995a) contrast markedly with our initial observation that the concentrations return to basal values after a few weeks on the high diet (Martin et al., 1994a). This result is probably due to quantitative differences between these studies in the composition of the high (lupin-supplemented) diet. In the more recent experiments, twice as much lupin grain was used, and hence more protein and energy, and this led to sustained body growth, compared with the diet used by Martin et al. (1994a) with which body weight reached a plateau before the end of the experiment. Although this explains the sustained high FSH concentrations in recent experiments, it also supports our previous contention that LH and FSH respond differently to nutritional factors in intact rams fed a diet lower than maintenance (Martin et al., 1994a; Hötzel et al., 1995a). Since GnRH stimulates LH and FSH secretion by the pituitary gland (Lincoln, 1978), it appears that nutrition affects the pituitary mechanisms controlling FSH release. These observations cannot be explained by effects of diet on inhibin or testosterone secretion, because the production of these hormones does not appear to be affected by diet (Martin et al., 1994a; Hötzel et al., 1995a) and because similar effects are evident in castrated rams given exogenous inhibin and testosterone (Tjondronegoro et al., 1996). Alternatively, diet might alter FSH concentrations independently of changes in GnRH by affecting concentrations of activin, a factor that has been implicated in the paracrine regulation of FSH secretion (Carroll et al., 1991; Farnsworth, 1995).

Within each immunization group, diet did not affect basal or LH-stimulated testosterone concentrations, reinforcing previous observations that nutrition does not affect steroidogenesis, despite the effects on testicular mass (Martin et al., 1994a; Hötzel et al., 1995a). Immunization against GnRH decreased peripheral concentrations of testosterone compared with control rams, but the lack of difference between the groups when testosterone was corrected for LH concentrations indicates that this was due to the lower LH stimulus in rats immunized against GnRH compared with control rats, rather than lower rates of steroidogenesis, suggesting that immunization against GnRH did not affect Leydig cell size or function. The effects of immunization against GnRH on steroidogenesis have been described in rats, in which lower testosterone secretion was associated with a decrease in the activity of 3β-hydroxysteroid dehydrogenase isomerase and reduced basal and hCG-stimulated activities of cholesterol side-chain

**Fig. 3.** (a) Mean (± SEM) plasma concentrations of FSH and (b) change in mean plasma FSH concentrations in mature Merino rams immunized against BSA and fed the high (○) or the maintenance (□) diet, and in rams immunized against GnRH–BSA and fed the high (●) or the maintenance (■) diet.
cleavage and 17α-hydroxylase (Chase et al., 1992). It is possible that a longer reduction of gonadotrophin concentrations than that achieved in this study is required to alter the steroidogenic capacity of the ram testis. In this study there was a trend for a lower testosterone response to LH in the GnRH-i rams fed the maintenance diet, suggesting that diet may interact with gonadotrophins in the regulation of steroidogenesis (Sharpe, 1984; Kerr and Sharpe, 1985; Kerr et al., 1988).

In conclusion, this study adds further support to the hypothesis that part of the effect of nutrition on testicular growth in mature rams is independent of changes in GnRH secretion. The relative effect of diet on testicular growth was of the same magnitude whether gonadotrophin concentrations were normal or lower than normal, suggesting an additive effect of GnRH-dependent and -independent mechanisms on the testes. The present results indicate that, although nutrition can alter testicular growth in the absence of changes in plasma gonadotrophins, the mechanism(s) by which it causes this growth depends on the concurrent presence of the gonadotrophins. Finally, the patterns of gonadotrophins observed in this study also suggest that FSH is likely to play a more important role than LH in this effect.

**Fig. 4.** Testosterone and LH secretion on day 70 in mature Merino rams immunized against GnRH-BSA and fed the high (■) or the maintenance (□) diet; and in rams immunized against BSA and fed the high (■) or the maintenance (□) diet. Responses were studied by measurement of (a) mean plasma concentrations of testosterone and (b) the ratio between mean plasma concentrations of testosterone and LH over 12 h or (c) the area under the testosterone curve and (d) the ratio between the areas under the testosterone and LH curves after an injection of oLH. Values are mean ± SEM and the areas are in arbitrary units.
This study could not have been contemplated without the generous assistance of the students and staff of the Animal Science Group (University of Western Australia) and the CSIRO Division of Animal Production. We are particularly indebted to K. L. Shepherd and M. A. Blackbery. The authors are also grateful to the National Institute of Diabetes, Digestive and Kidney Disease, the Center for Population Research of the National Institute of Child Health, and the Agricultural Research Service of the US Department of Agriculture, as well as the University of Maryland School of Medicine, for the reagents for the LH and FSH assays. They would like to acknowledge R. I. Cox (CSIRO Division of Animal Production, Prospect, NSW) for the donation of testosterone-3-CMO–HSA. M. J. Hötzel was supported by the Brazilian Research Council (CNPq). This work was funded by the Australian Research Council and the CSIRO Division of Animal Production.

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