Testicular and serum levels of inhibin and expression of inhibin subunit mRNAs are differentially affected by hemicastration in rats of various ages

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Abstract

Age-related short-term effects of hemicastration on testicular weight, serum FSH, immunoreactive inhibin, LH and testosterone, testicular levels of inhibin subunit mRNA expression, and bioactive and immunoreactive inhibin were studied in rats of 8, 15 and 22 days of age. Hemicastration led to an increased weight of the remaining testis after 24 h in 8- and 15-day-old rats, but not in 22-day-old rats. Serum FSH levels were elevated in all hemicastrated rats after 8 h. However, serum immunoreactive inhibin levels were decreased only after 72 h in 8-day-old rats and after 24 h in 15- and 22-day-old rats. Inhibin α-subunit mRNA expression was increased in the testes of hemicastrated rats of 8 and 15 days of age, whereas inhibin βB-subunit mRNA expression was elevated in the testes of 15-day-old rats but not in those of 8- and 22-day-old rats. The increase in α-subunit mRNA content per testis was caused by an increased concentration and increased testicular weight, whereas the increase in βB-subunit mRNA in the remaining testis paralleled the increased testicular weight, indicating that different mechanisms play a role in the regulation of these mRNAs. In 22-day-old rats, a transiently decreased expression of inhibin βB-subunit mRNA was observed 8 h after hemicastration. The increased inhibin α- and βB-subunit mRNA expression in 8- and 15-day-old rats did not result in increased testicular bioactive and immunoreactive inhibin content of the remaining testis, whereas in 22-day-old rats an increased immunoreactive inhibin content of the remaining testis was observed. These data indicate that efficiency of translation, post-translational modifications or transport from the testis play an important role in determining the final testicular content of inhibin.

In conclusion, the response of the remaining testis and the role of inhibin in the regulation of the pituitary-testis axis after unilateral castration depend on the age at which the animals are hemiorchidectomized.

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Introduction

The testicular glycoprotein hormone inhibin was first identified by its ability to inhibit the secretion of follicle-stimulating hormone (FSH) by the pituitary gland (for review see de Jong 1988). Under physiological circumstances in male rats this mechanism appears to be effective only in immature animals, since after castration an acute increase in FSH secretion occurred only in rats of 15 to 35 days of age, whereas in older rats only minor effects on FSH levels were found (Hermans et al. 1980, Rivier et al. 1989). Furthermore, passive immunization against inhibin caused increased levels of FSH in prepubertal but not in adult rats (Culler & Negro-Vilar 1988, Rivier et al. 1988). On the other hand, FSH was shown to stimulate production of inhibin by cultured Sertoli cells from immature rats (Bicsak et al. 1987), and daily administration of FSH to newborn rats stimulated testicular bioactive inhibin levels (Ultee-van Gessel et al. 1988). Finally, decreased serum FSH levels after hypophysectomy caused a larger decrease in inhibin α-subunit mRNA expression in immature rats than in mature rats (Krummen et al. 1989). Based on this evidence, it appears that regulation of FSH by inhibin and regulation of inhibin by FSH are only tightly interlinked during the prepubertal period.

Compensatory hypertrophy of the remaining testis 20 days after hemicastration of immature rats has been described (Moger 1977, Cunningham et al. 1978, Hochereau-de Reviers & Courtois 1978, Putra & Blackshaw 1982); this hypertrophy was associated with increased FSH levels (Brown et al. 1991). In addition, hemicastration of neonatal rats results in an increased amount of bioactive inhibin in the remaining testis after 1 to 6 weeks (Ultee-van Gessel & de Jong 1987). In the
present experiments, short-term effects of decreased serum inhibin levels on FSH and of elevated FSH levels on inhibin production were studied, using the model of hemicastrated immature rats of different ages. Furthermore, the possibility that regulation of the expression of inhibin subunit mRNAs contributes to the regulation of inhibin production in vivo was studied by measuring inhibin mRNA levels in the remaining testis.

Materials and Methods

Experimental procedure

To investigate the age-dependent effects of hemicastration on testicular inhibin content, Wistar rats (substrain R1) were hemicastrated at 8, 15 and 22 days after birth. The testes which were removed at the time of hemicastration were snap-frozen and used to generate the data on testicular mRNA and hormone levels at time zero. Groups of six to ten hemicastrated rats were killed by decapitation 8, 24 or 72 h after surgery, along with equal numbers of age- and body weight-matched sham-operated animals. Testes were snap-frozen in liquid nitrogen and stored at −80 °C until further processing. Five testes from 8- to 11-day-old rats were pooled for the isolation of RNA, and two pools of two and a half testes were used for the measurement of inhibin and testosterone. Testes from 15- to 18-day-old and 22- to 25-day-old rats were halved, and two to six halves were pooled for RNA isolation. The other hemi testes were used for hormone assay. Sera were collected and stored at −20 °C until hormone assays were carried out.

RNA isolation and analysis

RNA was isolated using the acid guanidium thiocyanate–phenol–chloroform extraction procedure described by Chomczynski & Sacchi (1987). The yield of total RNA per mg of tissue was not significantly different between groups, and amounted to 3·17±0·08 μg RNA/mg testicular tissue (mean±s.e.m.; n=57). Of each sample, 40 μg total RNA was subjected to Northern blotting, and hybridized with inhibin subunit cDNA probes as described earlier (Klaaj et al. 1992). The cDNA probes used were a 1·25 kb EcoRI fragment encoding the α-subunit (α7/pUC18), and a 1·5 kb EcoRI fragment encoding part of the βB-subunit (βB11/pUC18) of rat inhibin (Esch et al. 1987).

Intensity of hybridizing bands was measured by densitometric scanning as described previously (Klaaj et al. 1990). As a control for equal amounts of RNA, gels were stained with ethidium bromide to visualize ribosomal RNA bands. Hybridizing signals were not normalized against actin, since the expression of this mRNA in the testis changes with age (Slaughter et al. 1987).

In order to compare the expression of RNAs on different blots, a sample of 40 μg from a pool of total testicular RNA from 21-day-old rats was included on each blot, and the intensity of the hybridizing bands was related to the intensity of this standard.

Inhibin measurements

Testicular tissue was homogenized in a 5 ml Teflon–glass homogenizer in 1 ml phosphate-buffered saline (PBS). Homogenates were centrifuged at 100 000 g for 1 h at 4 °C in a Beckmann L5–65 ultracentrifuge using an SW60 rotor, and supernatants were collected. A volume of 50 μl was collected for the measurement of testosterone, and the remaining supernatant was incubated with an equal volume of dextran–coated charcoal suspension (1% Norit, 0·1% dextran T300 in PBS, pH 7·0) at 4 °C for 30 min in order to remove the steroids. Thereafter, charcoal was removed by centrifugation at 1500 g for 10 min and cytosols were collected, sterilized by filtration through a 0·2 μm filter (Schleicher & Schuell, Dassel, Germany) and stored at −20 °C until estimation of inhibin bioactivity and immunoreactivity.

Levels of immunoreactive inhibin in testis homogenates and serum were measured by radioimmunoassay as described by Robertson et al. (1988), using an antiserum against purified 32 kDa bovine follicular fluid (bFF) inhibin and iodinated 32 kDa bFF inhibin. These materials were kindly made available by Dr G Bialy (NICHHD, Bethesda, MD, USA). A charcoal-treated bFF preparation with an arbitrary potency of 1 U/μg protein was used as a standard (Grootenhuis et al. 1989). Testicular and serum immunoreactive inhibin was measured in two separate assays; the intra-assay variation coefficient amounted to 24·6% for testicular immunoreactive inhibin and 18·3% for serum immunoreactive inhibin.

The amount of bioactive inhibin was determined by an in vitro bioassay in which suppression of spontaneous FSH release from cultured rat pituitary cells was estimated (Grootenhuis et al. 1989). The bFF preparation described above was used as a standard. The precision index (λ) of these assays amounted to 0·145±0·006 (mean±s.e.m., n=5).

Measurement of FSH, LH and testosterone

FSH and luteinizing hormone (LH) were measured by radioimmunoassay (Grootenhuis et al. 1989), using the antibodies developed by Welschen et al. (1975). All results are expressed in terms of NIADDK-rat FSH-RP-3 and NIADDK-rat LH-RP-2. Testosterone was measured by radioimmunoassay, using the antiserum described by Verjans et al. (1973). All samples for each hormone were measured in one assay. Intra-assay coefficients of variation were 17·9% for FSH, 11·4% for LH and 3·5% for testosterone.
**Statistical analysis**

All data are presented as means ± S.E.M., except for results of Northern blotting of mRNA from 8-day-old hemicastrated rats which are single measurements of a pool of five testes. Data were analysed for each age group by two-way analysis of variance (ANOVA), with time and hemicastration as variables. When the effect of hemicastration was significant, significances of differences between control and hemicastrated rats at each time-point were assessed using the Student’s *t*-test. Differences were considered significant if *P*<0.05. Correlation between different parameters was determined using regression analysis.

**Results**

**Body and testis weights**

Body weights increased in an age-dependent manner throughout the experiment and were not affected by hemicastration at any age (not shown). In control and hemicastrated animals, testis weights increased with time and were correlated with body weights (controls: *r*=0.93, d.f.=64, *P*<0.0005; hemicastrated rats: *r*=0.94, d.f.=64, *P*<0.0005).

In hemicastrated rats of 8 and 15 days of age, a significant increase in the weight of the remaining testis occurred 24 h (P<0.0005 and P<0.01 respectively) and 72 h (P<0.01 and P<0.005 respectively) after hemicastration when compared with testis weights in control rats at the same time-points (Fig. 1). In contrast, no difference was found between the weights of the testes from 22-day-old hemicastrated and control animals.

**Serum hormone levels**

FSH levels remained constant with age in control animals (Fig. 2a), and were significantly elevated from 8 h after operation in hemicastrated rats of all ages when compared with control levels. However, the relative increase and the time-course of the increase differed between the three age groups.

Immunoreactive inhibin levels in serum were constant in control rats of 8–11 days of age, increased in rats from 15 days of age onwards, and decreased in rats from 22 days of age onwards (Fig. 2b). A significant decrease in immunoreactive inhibin was only observed in 8-day-old hemicastrated animals at 72 h after hemicastration (*P*<0.0005), whereas in 15- and 22-day-old rats significant decreases were found after 24 (*P*<0.025 and *P*<0.005 respectively) and 72 h (*P*<0.005 and *P*<0.05 respectively).

LH levels in control rats decreased with age from 2.4±0.1 ng/ml in 8-day-old rats to 0.5±0.1 ng/ml in 22-day-old rats. Hemicastration had no effect on LH levels (data not shown).

Testosterone levels were not measured in 8-day-old rats, since the amount of serum from these rats was limited. Testosterone levels in 15- and 22-day-old hemicastrated rats were not significantly different from levels in control rats (data not shown).

**Inhibin subunit mRNA levels**

Inhibin α-subunit mRNA levels (Fig. 3a) increased with time in control animals, when expressed as arbitrary units per testis. This increase was due to increasing testis weight,
since the expression of α-subunit mRNA per mg testis decreased with time. Compared with its expression in testes from intact rats, α-subunit mRNA expression was significantly increased in hemastraed rats of 8 and 15 days of age, from 24 h and 8 h onwards respectively. In contrast, no significant effect of hemastraed was observed in 22-day-old rats.

Combined levels of the 4-2 kb and 3-5 kb βB-subunit mRNAs are presented in Fig. 3b. Total inhibit βB-subunit mRNA expression per testis was constant in control rats of 8-11 days of age, the expression in 15-day-old rats was twice as high, and from 22 days onwards βB-subunit expression increased further (Fig. 3b).

No significant difference existed between βB-subunit mRNA expression in intact and hemastraed rats of 8 days of age, whereas a significant increase was found in hemastraed rats of 15 days after 24 and 72 h ($P<0.05$). In 22-day-old rats, a significant decrease in βB-subunit mRNA expression was found at 8 h after hemastraed. The ratio between 4-2 kb and 3-5 kb βB-subunit mRNA decreased with age from 2.20±0.37 in 8-day-old rats to 0.46±0.03 in 25-day-old rats. Hemastraed had no effect on the ratio in 8- and 15-day-old rats, but in 22-day-old hemastraed rats the ratio was significantly increased when compared with the ratio in controls (Table 1).
**Testicular hormone contents**

Testicular bioactive inhibin content in control rats increased with time in rats of 8–11 days of age, and was constant in 15- to 18-day-old rats and 22- to 25-day-old rats (Fig. 4a). No significant effect of hemastraion on bioactive inhibin content was found.

Testicular immunoreactive inhibin content increased with age until 22 days in control animals (Fig. 4b). No significant difference was observed between immunoreactive inhibin contents in control and hemastraion rats of 8 days and 15 days of age. The testicular content of immunoreactive inhibin increased time-dependently after hemastraion in 22-day-old hemastraion rats, but was only significantly higher than in control rats after 72 h ($P<0.0005$). Testicular levels of bio- and immunoreactive inhibin were correlated in testes from control ($r=0.589$, d.f.=37, $P<0.0005$) and hemastraion ($r=0.628$, d.f.=22, $P<0.005$) rats.

Testicular testosterone levels in control and hemastraion rats were not significantly different at any time-point studied (data not shown).
TABLE 1. Ratio between 4-2 kb and 3-5 kb βB-subunit mRNA expression in testes from control and hemicastrated rats of different ages at various times after surgery. Values are means ± S.E.M. of three to six pools of testes from two to six animals each, except for 8-day-old hemicastrated rats where one pool of testes from five rats was used.

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Time after surgery (h)</th>
<th>Ratio</th>
<th>Control</th>
<th>Hemicastrated</th>
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<tr>
<td>8</td>
<td>0</td>
<td>2.20 ± 0.37</td>
<td>2.20 ± 0.37</td>
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<tr>
<td></td>
<td>8</td>
<td>1.59 ± 0.16</td>
<td>1.49</td>
<td></td>
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<tr>
<td></td>
<td>24</td>
<td>2.72 ± 0.09</td>
<td>2.27</td>
<td></td>
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<tr>
<td></td>
<td>72</td>
<td>2.06 ± 0.12</td>
<td>2.07</td>
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<tr>
<td>15</td>
<td>0</td>
<td>0.73 ± 0.06</td>
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<td></td>
<td>8</td>
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<tr>
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<td>72</td>
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<td>8</td>
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<td>72</td>
<td>0.47 ± 0.03</td>
<td>0.54 ± 0.00*</td>
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</table>

*P<0.05 compared with control rats (ANOVA and Student's t-test).

Discussion

FSH levels and testicular hypertrophy

Changes in FSH levels with age in control rats are in agreement with those reported previously (Culler & Negro-Vilar 1988, Ackland & Schwartz 1991). The time-course of the increase of FSH in 15-day-old hemicastrated rats confirms the data of Brown & Chakraborty (1991).

The present results show that the testicular hypertrophy which has been observed by other authors at 20 days after hemicastration of prepubertal rats (Moger 1977, Cunningham et al. 1978, Hochereau-de Reviers & Courot 1978, Putra & Blackshaw 1982, Brown & Chakraborty 1991), is evident after 24 h in rats hemicastrated at the ages of 8 and 15 days. In contrast, no increase in testis weight was observed after hemicastration in 22-day-old rats up to 72 h after hemicastration.

Testicular hypertrophy has been associated with changes in serum FSH (Orth 1982, 1984), and has been attributed to two phenomena: an increase in the number of Sertoli cells per testis (Cunningham et al. 1978, Orth et al. 1984), or an increase in the number of germ cells per Sertoli cell (Putra & Blackshaw 1982). An increased number of Sertoli cells was only observed in rats hemicastrated when younger than 20 days (Cunningham et al. 1978, Putra & Blackshaw 1982); Sertoli cell division stops in normal rats at around 15-18 days of age (Steinberger & Steinberger 1971, Orth 1982, van Haaster et al. 1992). Apparently, FSH is able to stimulate the existing proliferation of Sertoli cells but cannot prolong the period during which proliferation takes place. The present study shows that FSH levels were significantly increased in hemicastrated rats of 8, 15 and 22 days of age after 8 h, but this resulted in a rapid increase of testis weight in 8- and 15-day-old rats only. The increased testis weight after 20 days described by others in rats hemicastrated at 20-25 days of age (Moger 1977, Putra & Blackshaw 1982) might therefore be caused by a mechanism which differs from the stimulation of Sertoli cell proliferation by FSH, e.g. by the stimulation of germ cell numbers in older rats. The role of inhibin and activin in this process remains unclear, since, in the present experiments, an increased amount of testicular immunoreactive but not bioactive inhibin was observed in 22-day-old hemicastrated rats.

Inhibin subunit mRNA expression

Age-dependent changes in the expression of inhibin α-subunit mRNAs in intact rats are in agreement with previous observations (Meunier et al. 1988, Keinan et al. 1989, Klaij et al. 1992): the concentration of inhibin α-subunit mRNA decreased with age but, together with an increase in testis weight, this resulted in an increasing testicular content. The expression of α-subunit mRNA per mg testis increased after hemicastration in 8- and 15-day-old rats. This is most probably caused by the increased FSH levels, since FSH has been shown to stimulate inhibin α-subunit mRNA expression in vitro (Toebosch et al. 1988, Keinan et al. 1989) by an increase in cyclic AMP (Klaij et al. 1990). In contrast, increased FSH levels did not cause an increased inhibin α-subunit mRNA expression in 22-day-old hemicastrated rats.

Levels of inhibin βB-subunit mRNA expression are in agreement with data obtained in a previous study (Klaij et al. 1992), in which a change in the ratio between the expression of 4-2 kb and 3-5 kb βB-subunit mRNA during testicular development was also observed. In contrast to the increased concentration of inhibin α-subunit mRNA after hemicastration, βB-subunit mRNA expression per mg testis did not change in 8- and 15-day-old rats after hemicastration. This is in agreement with the fact that FSH does not affect βB-subunit mRNA expression in cultured Sertoli cells (Toebosch et al. 1988, Klaij et al. 1990). The increase in testicular βB-subunit mRNA content can be explained on the basis of increased testis weight, indicating that inhibin α- and βB-subunit mRNA expression are differentially regulated in vivo. The transient decrease in βB-subunit mRNA expression in 22-day-old hemicastrated rats might have been caused by an effect of FSH on intracellular calcium levels (Grasso et al. 1991); increased calcium levels suppress βB-subunit mRNA expression in cultured Sertoli cells (Klaij et al. 1992). Conversely, Feng et al. (1989a) have shown that hypophysectomy results in increased inhibin βB-subunit mRNA concentration and content, possibly by decreasing FSH levels.

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The functional difference between the two $\beta$B-subunit mRNAs is not known. Although only one $\beta$B-subunit gene exists in the rat (Feng et al. 1989b), the two $\beta$B-subunit mRNAs originate from initiation of transcription at alternative transcription initiation sites (Feng & Chen 1993), and it is not clear whether this results in different open reading frames in the two mRNAs. Further investigation of the mRNAs is necessary to determine which differences exist between the two $\beta$B-subunit mRNAs and whether translation of the mRNAs results in different protein products. The ratio between the 4·2 kb and 3·5 kb $\beta$B-subunit mRNA changes with age, and may be indicative of the state of maturation of the Sertoli cells. The increased FSH levels after hemastraion in 22-day-old rats might delay maturation, as suggested by the relative increase in the ratio after hemastraion in rats of this age.

**Bioactive and immunoreactive inhibin in testes and serum**

The increased expression of inhibin $\alpha$- and $\beta$B-subunit mRNA in 8- and 15-day-old hemastraion rats was expected to cause increased testicular levels of bioactive and immunoreactive inhibin. Surprisingly, the testicular bioactive inhibin content was unchanged in 8- and 15-day-old rats. Immunoreactive inhibin was unchanged in 8-day-old hemastraion rats, and did not change systematically in 15-day-old rats. The reason for these
observations might be found in an increased secretion of inhibin from the testis, for plasma inhibin levels were decreased to a lower extent than would be expected on the basis of the initial short biological half-life of inhibin (Robertson et al. 1988, Woodruff et al. 1993). The blood-testis barrier has not yet been formed in 8- or 15-day-old rats (Setchell et al. 1988, Russell et al. 1989), and the secretion of inhibin into the circulation is therefore not restricted at these ages. An increased amount of testicular immunoreactive but not bioactive inhibin was observed in 22-day-old hemicastrated rats; this increased amount was not associated with a higher level of α-subunit mRNA expression. A possible explanation might be an increased translation rate of α-subunit mRNAs. Furthermore, altered post-translational modification of inhibin might take place in hemicastrated rats and might cause altered protein stability, as has been described for glycosylation of LH after castration (Keel & Grotjan 1985), where discrepancies have also been described between gonadotrophin mRNA levels and protein levels in castrated rats at certain ages (Pakarinen & Huhtaniemi 1992). The same explanations might be applicable for the observation of decreased ratios between bioactive and immunoreactive inhibin (B/I ratios) in the testes of 15- to 22-day-old rats as compared with B/I ratios in 8-20 day-old rats.

The decrease in serum inhibin levels does not precede the increase in FSH levels. This does not agree with the theory that increased levels of FSH in hemicastrated animals are caused by decreased levels of inhibin. However, it is not known whether the immunoreactive inhibin which is measured in the serum also is bioactive, since the radioimmunoassay used is specific for the α-subunit of inhibin (Robertson et al. 1989, Schneyer et al. 1990) and α-subunit containing proteins different from bioactive inhibin can be secreted by the testis (de Winter et al. 1992). Furthermore, a small decrease in bioactive inhibin levels might be sufficient to cause increased secretion of FSH from the pituitary.

It is concluded from this study that the effects of hemicastration on different testicular functions are related to the age at which the animals are hemicastrated. Short-term hypertrophy and effects on inhibin subunit mRNA expression occurred only in hemicastrated rats of 8 and 15 days of age. Testicular bioactive and immunoreactive inhibin content were not affected at these ages, probably because of increased transport from the testis.

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Inhibin and inhibin mRNA expression in hemicastrated rats


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