Plasma testosterone levels and Δ^5 -3 β -hydroxysteroid dehydrogenase activity in the testis of the rat following prolonged exposure to stress

Irina Pollard, J. R. Bassett and Jean M. P. Joss

School of Biological Sciences, Macquarie University, North Ryde, New South Wales, Australia 2113

Summary. Exposure of male CSF rats to a signalled, unpredictable 60-day stress regimen induced a significant elevation in circulating testosterone levels at Days 1 and 60 which was abolished by castration. Small morphological changes were seen in the Leydig cells in the early days of stressing. A significant increase in 3 β -HSD activity was seen by 5 days of stress, indicating increased steroidogenic activity.

Introduction

Exposure to a stress procedure may elicit a multiplicity of hormonal responses which, through their metabolic effects, are interdependent and can lead to disturbed physiological changes. Pollard, Bassett & Cairncross (1976) described an ultrastructural study of the adenohipophysis in stressed rats, in which the cellular activity of the corticotrophs correlated well with the circulating levels of corticosterone and the gonadotrophs showed marked morphological changes. Both the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) gonadotrophs showed intense secretory activity during the initial 10 days of stress. By 20 days, gonadotrophic activity returned to a control level, but there was then an inhibition of activity at 60 days.

Plasma testosterone levels have been reported to change following exposure to stressful situations, but the data appear to be contradictory and therefore difficult to interpret. For example, during intense physical exercise plasma testosterone levels have been reported to increase (Dessypris, Kuoppasalmi & Adlercreutz, 1976), decrease (Sutton, Coleman, Casey & Lazarus, 1973), or remain unchanged (Lamb, 1975). It is, however, accepted that stressors do elicit changes in testicular secretion, and the type of stress used could have caused different stress-induced changes in testosterone concentrations.

In this report the effects of prolonged stress on testicular secretion of testosterone were examined and correlated with ultrastructural changes in the Leydig cells of rats.

Materials and Methods

Animals and experiments

The male CSF rats were 87–93 days old when used. The animals were housed in groups of 3 under conditions of constant temperature and humidity ($21 \pm 0.5^\circ\text{C}$, 46% humidity) and with 12 h light (20:00–08:00 h)/24 h, beginning at least 14 days before the experiment and continuing throughout. Food and water were always available.

The experimental animals used for the testosterone measurements and ultrastructural studies were stressed for 1, 5, 10, 20, 40 and 60 consecutive days and rats for study of the activity of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in the Leydig cells were stressed for 1, 3, 5, 10, 20, 40 and 60 days. The stress procedure used was that described in detail by Bassett, Cairncross & King (1973). Animals were placed in automated one-way avoidance boxes (Lafayette Model No. 85200). An escape platform was available to the animal via an automated moving partition. A light-conditioned stimulus of 2 W was located on the wall of the grid chamber opposite the escape platform. The unconditioned stimulus was delivered by a generator-scrambler through the grids as a 2 mA, 50 pulses/sec square wave. Each rat was placed on the platform at the start of the treatment session. On each trial the conditioned stimulus started 4 sec before the animal was pushed by the movable partition from the platform onto the grid and the unconditioned stimulus began. The movable partition was then immediately retracted and the animal was able to jump from the grid to the re-exposed platform with a minimum latency of 0.3 sec. The unconditioned stimulus was stopped by the return of the animal to the platform. This irregular signalled foot-shock procedure is proposed as having a large psychological component (Bassett *et al.*, 1973). Each day the rats were subjected to 7 randomly timed shock treatments. Immediately after the last session, the animals were killed by cervical dislocation and exsanguinated. The blood samples were collected and the plasma was frozen. All samples were collected in the morning between 9:00 and 12:00 h in order to minimize diurnal variations.

This treatment procedure was repeated with castrated animals. Rats were castrated under ether anaesthesia and after 15 days one group of castrated animals was stressed for 1, 5, 10, 20, 40 or 60 days and the other group was left unstressed (controls). The controls were killed at the same time periods. There were 6 stressed and 3 control animals/time period.

Testosterone assay

Testosterone was assayed by the radioimmunoassay kit supplied by New England Nuclear (Biomedical Assay Laboratories). Testosterone was extracted from plasma using dichloromethane; no chromatographic separation was carried out. The freeze-dried antiserum is prepared in rabbits against testosterone-3-oxime-bovine serum albumin. Cross-reactivity at 50% displacement only occurred to any appreciable extent with dihydrotestosterone (100%). Unbound [1,2-³H(N)]testosterone was removed from the incubation mixture by using dextran-coated charcoal. The sensitivity of the assay was 0.01 ng/ml and the inter- and intra-assay variation were each <10%.

Electron microscopy

The testes from 5 stressed and 2 control animals from each group were rapidly removed and placed in 4% glutaraldehyde in 0.1 M-sodium cacodylate buffer for 2 h at 5°C. The tissues were then post-fixed in 2% osmium tetroxide for 1.5 h, dehydrated, and embedded in Araldite (CIBA). Silver sections were then stained by the triple stain method modified from Soloff (1973). The sections, which were mounted on copper grids, were treated with a 0.9% KMnO₄ solution buffered at pH 6.5 with phosphates for 2 min. They were then treated with uranyl acetate saturated in 50% ethanol for 3 min, followed by 0.2% lead citrate solution for 3 min. This method of staining increased electron density of the membrane systems in particular, which greatly improved cellular definition. The grids were examined in a JEOL 100CS electron microscope.

Assay for 3 β -HSD

Immediately after the last stress session rats from the control and stressed groups were killed and the testes were rapidly removed and frozen in liquid nitrogen.

Testicular 3 β -HSD was determined by the method of Pearse (1972). The 3 substrates used were pregnenolone, dehydroepiandrosterone (DHA) or epiandrosterone because 3 β -HSD converts pregnenolone to progesterone (a more distant precursor of testosterone steroidogenesis) and DHA and epiandrosterone to androstenedione and androstenedione respectively (more immediately involved in testosterone metabolism). In the presence of NAD as a cofactor the blue reduction product of tetrazolium, formazan, marked the location and intensity of enzymic activity. The intensity of this blue colour was rated on an arbitrary scale from 0 (absent) to 5 (intense) by visual examination of the slides.

Results

Testicular weight

No significant changes in wet weights of the testes were observed for stressed and control rats over the entire 60-day period. The mean $\pm$ s.e.m. wet weight values were 1.68 ± 0.06 and 1.70 ± 0.08 g for the right testes of stressed and control rats respectively.

Plasma testosterone

The results are given in Table 1. A one-way analysis of variance showed no significant difference between the control groups (intact or castrated) over the duration of the experiment ($F = 0.37$, d.f. 5, 17; $P > 0.05$) so the control groups were pooled. The plasma testosterone levels for the stressed rats showed a biphasic pattern, being significantly high on Days 1 and 60. Circulating testosterone was very low in the castrated control rats and values in the stressed and castrated animals were not significantly different throughout the experiment (Table 1).

Table 1. The effects of prolonged exposure to stress on mean $\pm$ s.e.m. testosterone levels in intact and castrated rats (no. in parentheses)

Days of stress	Plasma testosterone levels (ng/ml)	
	Intact	Castrated
Pooled control	3.7 ± 0.1 (18)	0.08 ± 0.01 (18)
1	* 4.8 ± 0.5 (6)	0.09 ± 0.01 (6)
5	4.2 ± 0.3 (6)	0.10 ± 0.01 (6)
10	3.9 ± 0.3 (6)	0.11 ± 0.005 (6)
20	3.4 ± 0.1 (6)	0.07 ± 0.005 (6)
40	3.4 ± 0.4 (6)	0.10 ± 0.01 (6)
60	* 4.4 ± 0.3 (6)	0.07 ± 0.005 (6)

* Significantly different from the pooled control value, $P < 0.02$ (unpaired t tests).

Leydig cell ultrastructure

The fine structure of the Leydig cells of control animals was similar to that described in the literature (Lacy & Pettitt, 1969). In general, stress did not cause great cytological alterations and no pathological signs were observed. However, small ultrastructural changes were observed after 5 days of stress. By Day 5 the Leydig cells showed an increase of both smooth and rough endoplasmic reticulum as well as free ribosomes and this was more marked after 10 days of stress when there was also a depletion of the lipid droplets, both in number present and size of the individual droplets, many being collapsed and crenated in appearance. By 10 days many of the mito-

chondria had changed from a spherical or oval shape with closely packed tubular cristae, as seen in the control animals, to structures which had enlarged and showed cavities, broken cristae and dense inclusions.

After 20 days of stress most of the above changes were still observable but to a lesser extent. By 40 and 60 days of stress, the lipid droplets had been replenished and the mitochondria and other cellular organelles were normal.

Activity of 3 β -HSD

The 3 β -HSD activity appeared to be strictly limited to the Leydig cells, and there was no trace of activity in the tubular cells. Formazan deposits of various intensities were found in all sections incubated with substrate whilst the sections lacking substrate had no formazan deposits (Pl. 1, Figs 1–4). As shown in Table 2 there was a significant increase in the density of formazan deposits with DHA and epiandrosterone by 3 days of stress and by 5 days all three substrates gave significantly elevated formazan deposits. The enzyme activity towards DHA remained elevated for 10 days of stress before falling to control levels. When pregnenolone and epiandrosterone were used as substrates, formazan deposits fell to the control level by 10 days.

Table 2. Mean $\pm$ s.e.m. activity (scored 1–5, see ‘Methods’) of Δ^5 -3 β hydroxysteroid dehydrogenase (3 β -HSD) in the testes of rats exposed to stress (6 rats/group, 3 rats/control group)

Days of stress	Substrate					
	Pregnenolone		Dehydroepiandrosterone		Epiandrosterone	
	Stress	Control	Stress	Control	Stress	Control
1	1.0 $\pm$ 0.4	0.3 $\pm$ 0.3	2.4 $\pm$ 0.2	1.7 $\pm$ 0.3	3.6 $\pm$ 0.2	3.3 $\pm$ 0.7
3	2.0 $\pm$ 0.4	0.7 $\pm$ 0.2	2.6 $\pm$ 0.2*	1.3 $\pm$ 0.3	4.6 $\pm$ 0.2*	3.0 $\pm$ 0.6
5	2.3 $\pm$ 0.2*	0.7 $\pm$ 0.3	4.0 $\pm$ 0.3*	2.3 $\pm$ 0.3	4.8 $\pm$ 0.3*	3.0 $\pm$ 0.6
10	1.8 $\pm$ 0.8	0.3 $\pm$ 0.3	3.0 $\pm$ 0.3*	1.3 $\pm$ 0.3	4.4 $\pm$ 0.7	3.7 $\pm$ 0.3
20	0.6 $\pm$ 0.2	0.3 $\pm$ 0.3	1.4 $\pm$ 0.4	1.3 $\pm$ 0.3	3.6 $\pm$ 0.4	3.3 $\pm$ 0.3
40	0.6 $\pm$ 0.2	0.7 $\pm$ 0.3	1.8 $\pm$ 0.2	1.7 $\pm$ 0.3	3.0 $\pm$ 0.3	2.3 $\pm$ 0.3
60	0.8 $\pm$ 0.4	0.3 $\pm$ 0.3	2.0 $\pm$ 0.4	2.3 $\pm$ 0.7	4.2 $\pm$ 0.6	3.3 $\pm$ 0.7

* Values significantly different from the respective control value, $P < 0.05$ (unpaired t tests).

Discussion

The stress-induced changes in testicular function, as reflected in plasma testosterone levels, correlate well with morphological change in the Leydig cells. The rises in plasma testosterone evident at Days 1 and 60 were completely abolished in castrated animals subjected to a similar regimen, demonstrating that the testosterone concentrations in the stressed animals were due to testosterone secreted by the testis and not by the adrenal gland. The range of circulating levels of testosterone for both the intact and castrated animals is in good agreement with those of other investigators (Verjans, van der Molen & Eik-Nes, 1975; Pujol, Bayard, Louvet & Boulard, 1976).

The described ultrastructural changes in the Leydig cells are all indicative of augmented secretory activity. The precise role of the lipid droplets in steroidogenesis is still not clear but it is thought that the lipid droplets in the Leydig cells function as storage depots of precursors used in the synthesis of androgens (Connell, 1976). Other investigators have observed that the droplets are rapidly depleted after stimulation by LH or hCG (Aoki & Massa, 1975). It has previously

PLATE 1

Micrographs of testes from rats subjected to 3 days exposure to the stressor.

Fig. 1. Control section lacking substrate (score 0).

Figs 2–4. Arbitrary values of enzyme activity with dehydroepiandrosterone as precursor of scores 2, 4 and 5 respectively. $\times 98$.

(Facing p. 104)

been shown (Pollard *et al.*, 1976) that in animals stressed for 10 days there was an extensive increased activity in the LH gonadotrophs, presumed indicative of pituitary secretion in the absence of an available assay for plasma gonadotrophins, but by 60 days the activity exhibited was below that of the controls. The extension of the rough endoplasmic reticulum together with free ribosomes in the Leydig cells in the present study is considered to be a reflection of the increased synthesis of smooth endoplasmic reticulum following demands of increased androgen synthesis. The observed mitochondrial changes at 10 days may also be a response to high circulating levels of LH. Gallon & Dufaure (1975) described similar alterations to the mitochondrial ultrastructure in rat luteal cells incubated with LH. Mitochondrial conversion of cholesterol to pregnenolone appears to be a rate-limiting step in the steroid synthesis of the testis and gonadotrophins might stimulate mitochondrial activity for pregnenolone production (Van de Vusse, Kalkman, Van Winsen & van der Molen, 1975). The reason for the increased plasma testosterone concentrations at Day 60 of stress is uncertain since no mitochondrial alterations were observed at this time (present study) and there were reduced ultrastructural changes in the LH gonadotrophs (Pollard *et al.*, 1976).

In the psychologically conditioned animal the hormonal profile may only represent alterations in energy metabolism. Plasma testosterone is significantly increased after strenuous running exercise and the effect is more pronounced in the fit runner than the non-fit runner (Kuoppasalmi, Naveri, Rehunen, Härkönen & Adlercreutz, 1976). Sutton *et al.* (1973) found that physical exercise raised serum androgens and that this rise was independent of serum LH values, perhaps because of reduced hepatic clearance of androgens. A decreased clearance of testosterone could be responsible for the elevated plasma testosterone in the rats of the present study which had habituated to the psychological stressor by 60 days (Pollard *et al.*, 1976).

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