Testosterone-dependent induction of metallothionein in genital organs of male rats

Chiharu TOHYAMA*¶, Junko S. SUZUKI*†, Shino HOMMA*, Mika KARASAWA‡, Toshio KUROKI‡, Hisao NISHIMURA§ and Noriko NISHIMURA||

* Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba 305, Japan, † Department of Biology, Kyoritsu College of Pharmacy, Shiba-koen, Minato-ku, Tokyo 113, Japan, ‡ Institute of Medical Science, University of Tokyo, Shirokane-dai, Minato-ku, Tokyo 108, Japan, § School of Human Sciences, Aichi Mizuho University, Toyota 470-03, Japan, and || Animal Production Division, CSIRO, Prospect, NSW 2148, Australia

Metallothioneins (MTs) are a group of cysteine-rich heavy-metal-binding proteins. We have investigated MT gene expression in the ventral and dorsolateral lobes of the prostate and coagulating gland of male Wistar rats. In intact rats, both MT mRNA and MT were present in the dorsolateral lobe and coagulating gland but not in the ventral lobe. Orchidectomy caused involution of the above organs, and both MT mRNA and MT were considerably decreased or became undetectable. An injection of testosterone propionate into orchidectomized rats

restored not only the size of these organs, but also MT mRNA and MT concentrations, particularly in the dorsolateral lobe and coagulating gland. In the dorsolateral lobe, no selective uptake of Zn^{2+} preceding the increase in MT was observed, suggesting that Zn^{2+} ions are not associated with the increased expression of the MT gene. The present result suggests that of the male auxiliary genital organs, the dorsolateral lobe and coagulating gland, but not the ventral lobe, contain MT, the biosynthesis of which is regulated by testosterone.

INTRODUCTION

Metallothioneins (MTs) are cysteine-rich low-molecular-mass proteins (approx. 7 kDa) that are found in various tissues from different animal species. Their gene expression and biosynthesis are regulated by heavy metals such as cadmium, zinc and copper as well as by endogenous factors such as cytokines and steroid hormones. Specific steroid hormones shown to regulate MT are glucocorticoid and progesterone (see the review by Bremner [1]) and vitamin D₃ [2]. MTs are considered to play a role in the detoxification of heavy metals and in the homeostatic regulation of essential metals such as zinc and copper, and have recently been proposed to participate in scavenging oxygen free radicals (see reviews in refs. [3–6]).

Male genital organs, such as testis and prostate, are susceptible to acute and chronic cadmium insult, resulting in necrotic changes in germ cells and tumorigenesis respectively (see reviews, in refs [7,8]. Although cadmium administration to rodents suppresses the synthesis of testosterone [9], no information is available on whether testosterone is involved in MT gene expression. We have thus investigated the effects of testosterone on MT gene expression in male genital tissues after orchidectomy, and as a result of testosterone treatment to testosterone-depleted rats.

MATERIALS AND METHODS

Reagents, chemicals and radioisotopes

Biotinylated goat anti-rabbit IgG (catalogue no. BA-1000), goat control serum (catalogue no. S-1000) and avidin–biotin peroxidase complex (ABC) kits were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). Protein assay kit was from Bio-Rad Co. (Richmond, CA, U.S.A.). Guanidine thio-

cyanate and CsCl were obtained from Fluka Chemie AG (Buchs, Switzerland) and Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.) respectively. Glyoxal (40 % ethanedial in aqueous solution) from Nakalai Tesque (Kyoto, Japan) was purified by using a mixed-bed ion-exchange resin (Bio-Rad AG 501-X8) until the pH of the eluate was neutral, and the purified glyoxal was stored at –20 °C in tightly capped tubes. [α -³²P]dCTP (3000 Ci/mmol) was purchased from Amersham-Japan (Tokyo, Japan). Other reagents were all analytical grade.

Animals

Male Wistar rats (7–8 weeks old), purchased from Shizuoka Animal Center, were orchidectomized under light ether anaesthesia and maintained for 15 days to ensure that plasma testosterone had reached the basal level. Plasma was collected by cardiac puncture at specified times after the surgical operation. The rats were injected with testosterone propionate, dissolved in sesame oil, in a single subcutaneous dose of 10.0 mg/kg body weight. Three groups of rats, i.e. intact rats, orchidectomized rats without any further treatment and orchidectomized rats injected with sesame oil, served as controls. Control and experimental groups consisted of five rats each. At the specified times after surgical operation or hormone administration, prostate (ventral and dorsolateral lobes), coagulating gland and liver were removed from the rats which were lightly anaesthetized with diethyl ether. Animals received humane care according to our institution's guidelines.

Immunohistochemical staining

For immunohistochemical evaluation, organs were fixed in 10 % buffered formalin and embedded in paraffin. Deparaffinized

5 μ m-thick tissue sections were stained by the ABC method with 3,3'-diaminobenzidine as a chromogen [10]. Controls were carried out by replacing the rabbit antiserum with control rabbit serum and by the use of rabbit antiserum that had been adsorbed with appropriate amounts of rat MT-2, another isoform that can completely cross-react with the antiserum [11]. Tissues were lightly stained with Haematoxylin.

Determination of MT

MT was determined by radioimmunoassay [12].

Isolation of total RNA and Northern-blot analysis

The isolation of total RNA from murine organs and Northern-blot analysis of MT in the tissues were performed as described previously [12]. Mouse MT-1 cDNA probe was a gift from R. Palmiter (Washington University, Seattle, WA, U.S.A.). In all experiments, at least duplicate gels were stained with ethidium bromide to ensure integrity of the RNA sample and to confirm that identical amounts of total RNA had been loaded on to each lane.

Other analyses

Plasma testosterone and corticosterone were determined with radioimmunoassay kits (Eiken Kagaku, Tokyo, Japan). Total protein in the tissue homogenate was quantified by the Bio-Rad dye-binding method with BSA as standard. Tissue Zn^{2+} concentrations were determined by atomic absorption spectroscopy (Hitachi 180-80 model, Tokyo, Japan) after digestion of tissues in an acid mixture [$HNO_3/HClO_4$ (3:1, v/v)].

Statistical analysis

For comparison of means of various parameters within different groups of rats, multiple comparison was carried out by one-way analysis of variance followed by Bonferroni's method. To compare means between two groups of rats, we used Welch's *t*-test. Means were considered to be significantly different at or below $P = 0.05$.

RESULTS

Effects of orchidectomy and testosterone on plasma testosterone concentrations

Orchidectomy resulted in a significant decrease in plasma testosterone concentration to 12% of the intact basal level (673 ± 81 ng/dl; mean $\pm$ S.E.M.; $n = 5$). In fact, the testosterone concentration had fallen dramatically to one-tenth of the normal concentration 24 h after orchidectomy, i.e. 45.8 ± 4.3 ng/dl and remained at this level until 15 days after surgery. No restoration of plasma testosterone levels after a prolonged time, which would reflect secretion of testosterone from the adrenal gland [13], was observed in the present study. Testosterone was administered on day 15 after orchidectomy by a single subcutaneous injection. Plasma testosterone concentration increased approximately 4-fold over the basal level by 24 h after the injection, and returned to basal levels by 72 h (537 ± 98 ng/dl); this was followed by a further decrease by 120 h (180 ± 21 ng/dl). Treatment with vehicle alone produced no significant change in plasma testosterone level over the 120 h experimental period.

As physical stress may induce MT biosynthesis, we determined plasma corticosterone concentrations. Plasma corticosterone

Table 1 Weight of male genital organs of rats in response to orchidectomy and testosterone treatment

Values are means $\pm$ S.E.M. for five rats except for coagulating glands from orchidectomized rats. Owing to atrophy after orchidectomy, coagulating glands were combined to make two specimens and utilized for various chemical analyses, and thus no statistical analysis could be performed. For comparison of means within different groups of rats, multiple comparison was carried out by one-way analysis of variance followed by Bonferroni's method. To compare means between two groups of rats, we used Welch's *t*-test.

Treatment	Weight (mg)		
	Ventral lobe	Dorsolateral lobe	Coagulating gland
Intact	263 $\pm$ 17	189 $\pm$ 9	109 $\pm$ 6
Orchidectomy	74.0 $\pm$ 3.9*	40.2 $\pm$ 1.5*	37.0
Orchidectomy + testosterone			
1 day	81.2 $\pm$ 5.3	78.0 $\pm$ 2.9†	21.4 $\pm$ 1.4
3 days	114 $\pm$ 7†	110 $\pm$ 3†	50.2 $\pm$ 2.5
5 days	154 $\pm$ 7†	158 $\pm$ 7†	51.0 $\pm$ 2.3
Orchidectomy + vehicle			
1 day	73.2 $\pm$ 4.6	56.8 $\pm$ 2.6‡	39.0
3 days	83.8 $\pm$ 3.4	42.0 $\pm$ 3.3	30.0
5 days	99.0 $\pm$ 4.4‡	47.0 $\pm$ 1.7	34.5

* Significantly different from intact rats ($P < 0.05$).

† Significantly different from corresponding values for orchidectomized rats injected with vehicle ($P < 0.05$).

‡ Significantly different from value for orchidectomized rats ($P < 0.05$).

Figure 1 MT mRNA levels in the ventral (PV) and dorsolateral (PDL) lobes of prostate and coagulating gland (CG) of rats in response to orchidectomy and testosterone

Unless specified, organs from three rats were combined to isolate total RNA. Because of atrophy of the coagulating gland after orchidectomy, 15 glands were used for a single lane (2, 3 and 4). To each lane, 25 μ g (or 10 μ g in the case of coagulating glands) of total RNA was applied. Lane 1, intact; lane 2, orchidectomy; lane 3, 24 h and lane 4, 48 h after sesame oil injection into orchidectomized rats; lane 5, 24 h and lane 6, 48 h after testosterone injection into orchidectomized rats.

fluctuated, but fell within the physiological concentration range: 45.6 ± 2.2 μ g/dl in the intact group; 50.9 ± 3.6 μ g/dl in the orchidectomy+vehicle group; 37.6 ± 2.2 μ g/dl in the orchidectomy+testosterone group. In another experiment, average levels of plasma corticosterone fluctuated within the physiological range, i.e. between 25.0 and 45.7 μ g/dl, for the first 10 days after orchidectomy. No effect of vehicle on plasma corticosterone levels was observed. These results show that MT was not induced by stress-mediated effect of orchidectomy.

Figure 2 Localization of MT in the prostate and coagulating gland of rats in response to orchidectomy and testosterone

(a) With preimmune serum, no specific immunostaining for MT was observed. (b) The lateral prostate from an intact rat shows strong MT staining in epithelial cells. (c) No MT was observed in the ventral lobe from an intact rat whereas strong MT staining was present in the lateral lobe. (d) Orchidectomy resulted in atrophy of the lateral lobe in which MT immunostaining was abolished. (e) On day 3 after testosterone administration the lateral prostate grew in size with reappearance of MT in the epithelium. (f) Weak MT staining was observed in the coagulating gland of intact rats. (g) Orchidectomy caused atrophy of the epithelium in which MT immunostaining could not be detected. (h) On day 3 after testosterone treatment, epithelial cells grew in size, coincident with the appearance of MT. Tissues were stained lightly with Haematoxylin. Bars = 50 μ m.

Table 2 MT levels in male genital organs of rats in response to orchidectomy and testosterone treatment

Values are means $\pm$ S.E.M. for five rats except for coagulating glands from orchidectomized rats (see also the footnote to Table 1). n.d., Not detectable (< 35 ng/mg of protein).

	MT concentration (ng/mg of protein)		
	Ventral lobe	Dorsolateral lobe	Coagulating gland
Intact	n.d.	473 $\pm$ 42	126 $\pm$ 13
Orchidectomy	50.6 $\pm$ 12.2	91.6 $\pm$ 10.2*	80.1
Orchidectomy + testosterone			
1 day	40.7 $\pm$ 11.1	110 $\pm$ 10	143 $\pm$ 8
3 days	48.8 $\pm$ 4.1	293 $\pm$ 29†	441 $\pm$ 18
5 days	39.1 $\pm$ 3.6	301 $\pm$ 23†	226 $\pm$ 17
Orchidectomy + vehicle			
1 day	50.4 $\pm$ 15.6	85.4 $\pm$ 8.2	57.2
3 days	75.2 $\pm$ 15.3	80.5 $\pm$ 5.4	73.6
5 days	70.5 $\pm$ 16.4	59.6 $\pm$ 4.3	83.7

* Significantly different from intact rats ($P < 0.05$).

† Significantly different from corresponding values for orchidectomized rats injected with vehicle ($P < 0.05$).

Table 3 Zn²⁺ levels in male genital organs of rats in response to orchidectomy and testosterone

Values are means $\pm$ S.E.M. for five rats except for coagulating glands from orchidectomized rats (see also the footnote to Table 1).

	Zn ²⁺ concentration (ng/mg of protein)		
	Ventral lobe	Dorsolateral lobe	Coagulating gland
Intact	79.4 $\pm$ 5.3	450 $\pm$ 52	124 $\pm$ 8
Orchidectomy	139 $\pm$ 21	264 $\pm$ 7*	101
Orchidectomy + testosterone			
1 day	115 $\pm$ 12	248 $\pm$ 11	234 $\pm$ 8
3 days	94.8 $\pm$ 7.4	220 $\pm$ 6	148 $\pm$ 2
5 days	111 $\pm$ 5	261 $\pm$ 25	131 $\pm$ 5
Orchidectomy + vehicle			
1 day	153 $\pm$ 23	241 $\pm$ 5	185
3 days	116 $\pm$ 18	284 $\pm$ 39	221
5 days	105 $\pm$ 10	278 $\pm$ 18	269

* Significantly different from intact rats ($P < 0.05$).

Tissue weight after orchidectomy and testosterone treatment

Orchidectomy resulted in a dramatic decrease in the weight of male reproductive organs, i.e. prostate (both ventral and dorsolateral lobes) and coagulating gland (Table 1). This decrease was partially reversed after a single subcutaneous injection of testosterone. This hormonal effect was particularly evident in the dorsolateral and ventral lobes but not significant in the coagulating gland (Table 1). The restoration of organ weight at day 5 was approximately 60% and 85% for the ventral lobe and dorsolateral lobe respectively.

MT mRNA levels after testosterone treatment

When equivalent amounts of total RNA (25 μ g) were analyzed by Northern blotting, MT mRNA was found to be abundant in

the dorsolateral lobe of the prostate but was not detectable in the ventral lobe. As the coagulating gland was very small, we used less total RNA (10 μ g) in each lane, but we were able to detect MT mRNA in this tissue from intact rats (CG in Figure 1, lane 1). Orchidectomy markedly decreased MT mRNA in the dorsolateral lobe (PDL in Figure 1, lane 2), but this was followed by a substantial increase in MT mRNA abundance in this tissue within 24 h of a testosterone injection, which persisted to 48 h after the injection (PDL in Figure 1, lanes 5 and 6). In the case of the ventral lobe, no increase in MT gene expression was observed as a function of testosterone injection (PV in Figure 1, lanes 5 and 6). In the coagulating gland (CG in Figure 1), the level of MT mRNA was not altered by orchidectomy or vehicle treatment, but had increased markedly 48 h after testosterone injection (CG in Figure 1, lane 6). No significant contribution of vehicle administration to MT mRNA induction was found in any of the three tissues (PV, PDL and CG in Figure 1; lanes 3 and 4 of each tissue). The above observations were confirmed in three independent experiments.

Immunohistochemical localization of MT in the prostate and other male genital tissues after testosterone treatment

Immunohistochemical controls using preimmune serum showed no MT in the epithelium, but the secreted material in the lumen produced a slight non-specific stain (Figure 2a). A specific antiserum against rat MT gave strong MT immunostaining, predominantly in the cytoplasm and nuclei of epithelial cells of lateral (Figure 2b) and dorsal (not shown) lobes of intact rats. The staining intensity for MT varied somewhat, and was much stronger in the lateral lobe than the dorsal lobe. Furthermore some cells contained MT in both the cytoplasm and nucleus whereas others contained MT only in the cytoplasm. Almost no staining was observed in the ventral lobe (Figure 2c). These observations are consistent with earlier findings in the rat prostate [10,14].

Orchidectomy caused atrophy of the prostate (Figure 2d): the size (thickness and width) of the columnar epithelium was dramatically decreased while the amount of connective tissue increased. Castration drastically reduced the MT immunostaining in the epithelium of the lateral (Figure 2d) and dorsal (not shown) lobes. Interestingly, on day 3 after testosterone administration, MT immunostaining appeared in the cytoplasm and/or nuclei of epithelial cells of the lateral (Figure 2e) and dorsal (not shown) lobes, but not in the ventral lobe (not shown).

In the coagulating gland, MT was found in the cytoplasm and/or nuclei of epithelial cells (Figure 2f), but was abolished by orchidectomy (Figure 2g). The connective tissue of this gland is well developed compared with the prostate, and it showed evidence of atrophy as a result of orchidectomy (Figure 2g). On day 3 after testosterone treatment, epithelial cells grew in size coincident with the appearance of MT (Figure 2h). When compared with coagulating glands from intact rats (Figure 2f), this MT immunostaining was more prevalent in the nuclei of epithelial cells (Figure 2h).

Tissue concentrations of MT after testosterone treatment

MT concentrations differed markedly among the male genital organs of intact rats (Table 2). The highest mean MT concentration (473 ng/mg of protein) was found in dorsolateral lobes compared with other tissues, i.e. coagulating gland (126 ng/mg of protein) and the ventral lobe (not detectable).

Orchidectomy produced a dramatic decrease in MT to approx. 20% and 50% of the intact level in the dorsolateral lobe and coagulating gland respectively. Virtually no difference in the amount of MT was observed in the liver or the ventral lobe.

On days 3 and 5 after testosterone administration, MT levels in the dorsolateral lobe were found to be significantly higher than those of the corresponding vehicle-treated groups of rats. On day 3, the level was approx. 60% of the intact level. In contrast, testosterone caused no increase in MT in the ventral lobe. In the coagulating gland, MT levels were increased approx. 5.5-fold by testosterone by day 3, reducing to 2.8-fold by day 5 when compared with orchidectomized rats (without vehicle administration).

Liver MT levels were not significantly altered by orchidectomy and/or testosterone administration, and were similar to the intact basal MT level (100 ± 4 ng of MT/mg of protein).

Tissue Zn^{2+} concentrations after testosterone treatment

Mean Zn^{2+} concentrations in the male genital organs from intact rats varied from 80 ng/mg of protein in the ventral lobe to 124 ng/mg of protein in the coagulating gland and 450 ng/mg of protein in the dorsolateral lobe (Table 3). They decreased significantly as a result of orchidectomy only in the dorsolateral lobe (Table 3).

No statistically significant changes in tissue Zn^{2+} concentrations as a function of testosterone administration were observed in the dorsolateral and ventral lobes when compared with corresponding vehicle-treated or orchidectomized rats (Table 3). In the coagulating gland, there appeared to be some increase in Zn^{2+} concentration on treatment with vehicle. The tissue Zn^{2+} concentrations after testosterone treatment appeared to be similar to or lower than those in corresponding vehicle-treated animals (Table 3).

In the liver, the mean Zn^{2+} concentration was 104 ng/mg of protein, and orchidectomy and/or testosterone administration had no effect.

DISCUSSION

Accumulated experimental evidence supports the notion that MT is present in male genital organs, such as testis and prostate, although there is still controversy about the existence of this unique metal-binding protein in these tissues [15]. In the testis and dorsolateral prostate in particular, constitutive expression of MT is considered to be high compared with other organs. Although the biosynthesis of MT has been reported to be regulated by steroid hormones such as glucocorticoid, female sex hormones and vitamin D_3 *in vivo* and/or *in vitro*, it is not clear how MT biosynthesis in the male auxiliary organs is regulated by testosterone. In the present study we have shown that testosterone causes a reversal of the effect of orchidectomy on MT induction in the dorsolateral lobes and coagulating gland. Although it was reported that Zn^{2+} ions were selectively taken up by the dorsolateral lobes of castrated rats after testosterone treatment [16], no significant uptake of Zn^{2+} was observed in any of the male genital organs examined in the present study. In the coagulating gland, vehicle administration after orchidectomy appeared to increase Zn^{2+} concentration, but no MT was induced under these conditions. However, MT was clearly induced by testosterone treatment. These data suggest that MT gene expression in the dorsolateral prostate and perhaps coagulating gland is specifically stimulated by testosterone.

Steroid hormone receptors have a common structure among different animal species, and have domains for DNA binding and ligand binding [17]. In the case of human steroid hormone receptors, the homology of the DNA-binding domain of various steroid hormone receptors relative to that of the glucocorticoid receptor is 94% of mineral corticoid, 91% for progesterone, 62% for oestrogen and 76% for androgen. Glucocorticoid-response elements have been found in promoter regions of human MT-II and mouse MT-I genes (see the review by Hamer [3]). Since the plasma testosterone concentration was markedly elevated after testosterone administration, there is the possibility that the glucocorticoid-response element reacted with the androgen receptor and upregulated MT gene transcription. However, this possibility is considered to be unlikely because other male auxiliary organs such as the ventral lobe of the prostate responded to testosterone for growth but not for MT biosynthesis, and because the liver, which has glucocorticoid hormone receptors, did not respond to testosterone under the present experimental conditions.

With respect to a possible stress response after orchidectomy, corticosterone has been found to increase MT content in primary rat hepatocytes at a concentration of 0.1 mM, but this is at least 100 times higher than the concentrations observed in the plasma of rats after severe stress [18]. Furthermore responses to glucocorticoid hormones may differ completely *in vitro* and *in vivo*, and different organs may respond to glucocorticoid hormones in a contrary manner [19,20]. However, since plasma corticosterone concentrations fluctuated within the physiological range in the present study, it is not likely that a stress response of any type is responsible for the appearance of MT in the male auxiliary organs examined.

Orchidectomy resulted in involution of the male auxiliary organs, i.e. the ventral and dorsolateral prostate and coagulating gland. This was markedly reversed by testosterone, which, as well as being known to be involved in hyperplasia, is required for significant cell proliferation in male auxiliary organs such as the seminal vesicle [21].

Male genital organs are extremely susceptible to cadmium insult, and cadmium is considered to be a potent carcinogen in various tissues including the prostate in experimental animal models [8]. The male auxiliary organs are under the control of testosterone, the synthesis of which is affected by cadmium administration in rats [9]. MT has been shown to play a detoxifying role against heavy-metal toxicity, particularly cadmium. Waalkes and Perantoni [22] reported that the prostate of rats contains a cadmium-binding protein, which is different from MT in terms of amino acid composition, and proposed that the absence of MT from this organ may be relevant to the susceptibility of the prostate to cadmium tumorigenicity. Although our present results on the absence of MT from the ventral lobe may support this notion, more importantly, it confirms earlier observations on the presence of MT in the dorsolateral lobes of the prostate [10,14,23]. Furthermore it is likely that a decrease in testosterone biosynthesis caused by exposure to cadmium aggravates the toxic effects of this metal on male auxiliary organs.

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