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Rat Testis during 2,5-Hexanedione Intoxication and Recovery

II. Dynamics of Pyrrole Reactivity, Tubulin Content, and Microtubule Assembly[1]

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Rat Testis during 2,5-Hexanedione Intoxication and Recovery. II. Dynamics of Pyrrole Reactivity, Tubulin Content, and Microtubule Assembly. BOEKELHEIDE, K. (1988). *Toxicol. Appl. Pharmacol.* **92**, 28–33. Charles River CD rats (200 g) were intoxicated with 1% 2,5-hexanedione (2,5-HD) in the drinking water for 5 weeks followed by a 17-week recovery period. Pyrrole reactivity of testis proteins increased early during intoxication and then returned toward normal during recovery. Testis tubulin content first increased as germ cells were lost and then fell over time while atrophy was maintained. Purified testis tubulin demonstrated a decreased nucleation time for microtubule assembly at 2 weeks, maintained this alteration throughout intoxication, and then returned to normal assembly kinetics during recovery. The assembly abnormality was accompanied by the presence of a unique crosslinked tubulin species. These findings support the hypothesis that alterations in Sertoli cell microtubules result in germ cell loss following 2,5-HD exposure.

2,5-Hexanedione (2,5-HD)[2] reacts with proteins in two sequential steps, first forming aromatic heterocyclic pyrroles with the ϵ-amines of lysine residues (DeCaprio *et al.*, 1982; Anthony *et al.*, 1983). Pyrrole formation is an irreversible chemical modification of a protein and a required step in the evolution of neurotoxic injury (DeCaprio and Jackowski, 1987; Genter *et al.*, 1987). In a subsequent oxygen-dependent reaction, these pyrroles are activated to form crosslinks. Hyperoxic conditions accelerated the development of 2,5-HD-induced neurotoxicity (Rosenberg *et al.*, 1987), indicating that an oxygen-dependent crosslinking reaction was rate limiting and required for nervous system injury.

We have described alterations induced by 2,5-HD in microtubules, a cytoskeletal component (Boekelheide, 1987a,b). Microtubules result from the self-assembly of tubulin, a 100-kDa dimeric protein. Intoxication with 2,5-HD accelerated the self-assembly of tubulin purified from rat testis. The effect of *in vivo* exposure to 2,5-HD upon microtubule assembly was mimicked by *in vitro* incubation of 2,5-HD with purified tubulin. Both testis tubulin purified from intoxicated rats and tubulin from naive rats treated with 2,5-HD *in vitro* contained a unique crosslinked tubulin dimer. The crosslinked tubulin dimer was produced in an oxygen-dependent reaction. *In vitro,* the amount of the crosslinked tubulin species correlated with the extent of altered microtubule assembly.

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[2] Abbreviations used: 2,5-HD, 2,5-hexanedione; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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The Sertoli cell is one target cell for the testicular injury induced by 2,5-HD (Chapin *et al.*, 1982, 1983; Boekelheide, 1988). Morphologically, microtubules are abundant cytoplasmic structures in the Sertoli cell (Fawcett, 1975). Sertoli cell microtubules appear to play a critical role in supporting and translocating germ cells (Russell *et al.*, 1981; Vogl *et al.*, 1983).

In this study, we have focused upon microtubule dynamics in rat testis during 2,5-HD intoxication and recovery. Atrophic testes, lacking germ cells and therefore relatively enriched in Sertoli cells, were also enriched in tubulin. 2,5-HD intoxication induced early changes in testis pyrrole content and microtubule assembly which returned to normal in a subsequent recovery phase. These biochemical studies complement the light microscopic observations reported in the companion article (Boekelheide, 1988).

MATERIALS AND METHODS

These biochemical experiments were performed with tissues obtained from Charles River CD rats (200 g) intoxicated for 5 weeks with 1% 2,5-HD (v/v) in the drinking water followed by a 17-week recovery period, as described in the companion article (Boekelheide, 1988). Determinations of tubulin content and pyrrole reactivity, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and tubulin purification were performed as previously described (Boekelheide, 1987a,b).

The microtubule assemblies used twice-cycled testis or brain tubulin freed of microtubule-associated proteins. The brain tubulin solutions were purified from groups of 5 rats throughout the time course of intoxication and recovery. Brain tubulin assemblies were performed in triplicate at 0.52 ± 0.01 mg/ml with 0.25 mM guanosine 5′-triphosphate at 37°C in 1 M sodium glutamate, pH 6.6. Appropriate control tubulin solutions were simultaneously assembled with each set of experimental tubulin preparations. The standard errors for these triplicate rat brain tubulin assemblies ranged from 1 to 11% of the mean (average, 5% of the mean). Groups of 15 rats, intoxicated in an identical fashion, were required for the testis tubulin assembly studies. Purified testis tubulin solutions from all six time points examined (weeks 0, 2, 5, 8, and 12 treated and 12 control) were assembled simultaneously to minimize variation. The simultaneous assembly of all samples was repeated in quadruplicate. The nucleation time was calculated by linear regression analysis of these quadruplicate assemblies, extrapolating back to a zero event time (considering each of the sequential assemblies as one unit of time on the abscissa). The correlation coefficient for these assemblies ranged from $r = 0.847$ to 0.990. Linear regression analysis corrected for a time-dependent change in assembly behavior.

The mean ± SE was calculated for all determinations involving multiple samples.

RESULTS

The samples for these biochemical studies were obtained from a 22-week time-course study consisting of a 5-week exposure to 1% 2,5-HD in the drinking water, followed by a 17-week recovery period without 2,5-HD consumption. Severe testicular atrophy, defined as the loss of greater than 50% of control testis weight, was present in all rats from weeks 7 through 22. The time-dependent changes in clinical neurotoxicity, body and testis weights, and the light microscopic testis histopathology were described in the companion article (Boekelheide, 1988).

Pyrrole Formation in the Testis

Crude testis supernatants were evaluated for pyrrole content in comparison with a dimethylpyrrole standard using Ehrlich's reagent. After 2 weeks of intoxication, pyrrole content reached a level of greater than 30 nmol of dimethylpyrrole equivalents per gram testis (Fig. 1). Pyrrole content remained in the range of 20–25 nmol of dimethylpyrrole for the rest of the intoxication phase and then decreased progressively in the recovery phase.

Changes in Testis Tubulin Content with Intoxication

Tubulin as the percentage of protein in testis crude supernatants was determined by col-

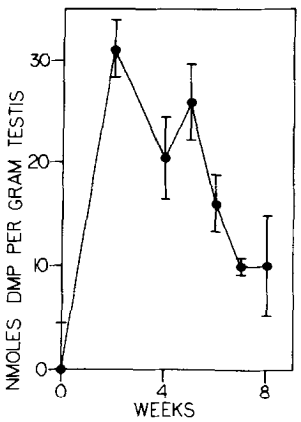

FIG. 1. Testis pyrrole reactivity increased during intoxication. Ehrlich's reaction was performed in triplicate on pooled testis crude supernatants during the first 8 weeks of the time-course study using dimethylpyrrole (DMP) as a standard. The pyrrole reactivity is expressed in equivalents of dimethylpyrrole present per gram decapsulated testis. The background pyrrole reactivity of the 0 week control group was subtracted from each experimental point.

chicine binding throughout the course of intoxication and recovery (Fig. 2). Testis tubulin content increased with germ cell loss. Tubulin was 3.3 ± 0.2% (weeks 0, 2, 4, and 5) of testis supernatant protein before atrophy and almost doubled to 6.2 ± 0.6% (weeks 7, 8, 10, 12, 16, and 22) in atrophic testes. Linear regression analysis of testis tubulin content in

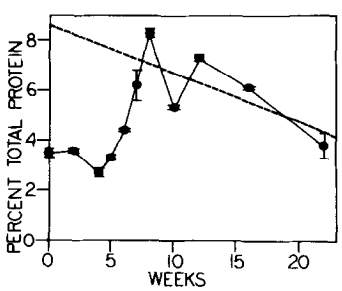

FIG. 2. Testis tubulin content, expressed as the percentage of total supernatant protein, was determined in triplicate on pooled testis crude supernatants. Linear regression analysis (dotted line) was performed for data from severely atrophic testes (weeks 7 to 22).

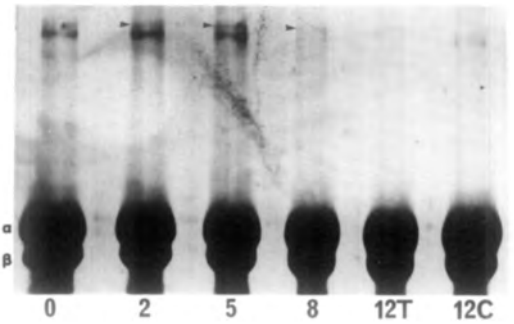

FIG. 3. SDS–PAGE analysis of purified testis tubulin from the time-course study. Testis tubulin was purified by DEAE-Sephacel binding and elution followed by two cycles of temperature-dependent assembly and disassembly. Samples were prepared from weeks 0, 2, 5, 8, and 12 treated (12T) and 12 control (12C) of the time-course study. A unique crosslinked tubulin species was seen at weeks 2, 5, and 8 (arrowheads).

atrophic testes (weeks 7, 8, 10, 12, 16, and 22) indicated a progressive loss of tubulin with time of atrophy (Fig. 2, dotted line, $r = -0.701$).

Analysis of Purified Testis Tubulin from Intoxicated Rats

Tubulin was purified from the testes of groups of 15 rats at selected time points (0, 2, 5, 8, and 12 weeks) during the course of intoxication and recovery. A unique high-molecular-weight band identified by SDS–PAGE analysis corresponded to a crosslinked tubulin dimer (Fig. 3), as previously described (Boekelheide, 1987a,b). The quantity of the crosslinked species was high during intoxication and then decreased with recovery.

The purified tubulin was analyzed for assembly behavior by observing the change in optical density at 350 nm which occurs when soluble tubulin forms microtubules (Johnson and Borisy, 1977). Intoxication decreased the nucleation time of assembly, which is the time required for the reaction to achieve

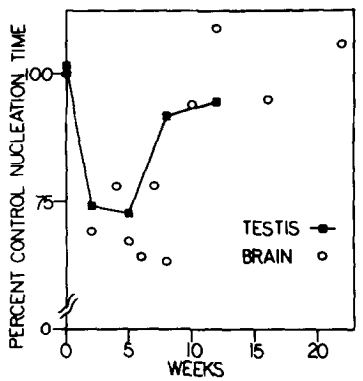

FIG. 4. 2,5-HD decreased the nucleation time of purified testis tubulin. Purified testis tubulin (see Fig. 3) was assembled at 0.55 ± 0.01 mg/ml at 37°C with 0.25 mM guanosine 5′-triphosphate in 1 M sodium glutamate, pH 6.6. The nucleation time for testis tubulin assembly (solid boxes) decreased by 2 weeks of 2,5-HD exposure and then gradually returned to normal after the 5-week intoxication period. The testis tubulin assemblies were normalized to the 12-week control assembly sample. Tubulin purified from brain (open circles) demonstrated a similar alteration in assembly behavior.

maximal velocity of assembly (Fig. 4). The decrease in nucleation time was evident after 2 weeks of intoxication, remained low throughout the intoxication phase, and then gradually returned to normal after withdrawal of 2,5-HD. Assemblies of purified tubulin from the brain of 2,5-HD-intoxicated rats demonstrated a similar decrease in nucleation time and dynamics of recovery (Fig. 4).

DISCUSSION

In previous studies of 2,5-HD intoxication, the earliest identified biochemical markers of injury were decreases in the activity of Sertoli cell specific enzymes after 3 weeks exposure to 1% 2,5-HD in the drinking water (Chapin *et al.*, 1982). Alterations in lipid metabolism have been identified as a late consequence of intoxication (Gillies *et al.*, 1981). The earliest morphological evidence of toxic injury occurred in the form of Sertoli cell vacuolation after 3–4 weeks of intoxication (Chapin *et al.*, 1983; Boekelheide, 1988). These experiments have examined dynamic aspects of pyrrole reactivity, tubulin content, and microtubule assembly occurring during 2,5-HD exposure and recovery. We have demonstrated significant alterations in testis microtubule assembly kinetics by 2 weeks of intoxication, before germ cell loss. These biochemical experiments complement the morphological data described in the companion article (Boekelheide, 1988).

Testis pyrrole reactivity reached a maximum of 30 nmol of dimethylpyrrole equivalents per gram testis after 2 weeks of 2,5-HD intoxication, the earliest time point of observation. This peak of pyrrole reactivity was followed by a plateau of 20–25 nmol of dimethylpyrrole equivalents per gram testis which was maintained throughout intoxication. A higher peak of pyrrole reactivity with a similar plateau level has been reported for brain during daily oral dosing of hens with 2,5-HD at 200 mg/kg (DeCaprio *et al.*, 1983).

During 2,5-HD intoxication and recovery, testis tubulin content increased with the onset of atrophy. We propose that this increase in testis tubulin content was due to a relative increase in the contribution of Sertoli cells to the supernatant protein. Previous ultrastructural investigations, some of which used microtubule disrupting agents, have documented the presence of a rich microtubule network of fundamental importance to Sertoli cell structure and function (Fawcett, 1975; Parvinen *et al.*, 1978; Russell *et al.*, 1981; Vogl *et al.*, 1983; Soderstrom and Roytta, 1986). Our own studies with purified preparations of Sertoli cells isolated from 18 day old rats by enzymatic digestion and differential centrifugation have indicated a high tubulin content in these cells.[3] Therefore, with atrophy and germ cell loss, Sertoli cells became the major cellular constituent in the testis. The increased tubulin content,

[3] R. E. Chapin and K. Boekelheide, manuscript in preparation.

measured as the percentage of supernatant protein, reflected this Sertoli cell enrichment.

During recovery, there was a progressive decrease in testis tubulin content from the high level achieved at the onset of atrophy. This decrease in tubulin content cannot be explained by restitution of germ cells, since both testis weight and morphology indicated continued germ cell depletion (Boekelheide, 1988). In addition, histopathological examination indicated an apparently intact, although morphologically altered, Sertoli cell population. Therefore, the time-dependent loss of tubulin in atrophic testes likely represented a cytoskeletal "accommodation" of Sertoli cells to the absence of germ cells. This observation suggests that microtubules within Sertoli cells perform supportive roles dedicated to germ cells.

Altered testis microtubule assembly was detected at the earliest time point of observation, 2 weeks after the beginning of 2,5-HD intoxication. The alteration consisted of an early onset of microtubule assembly resulting in a decreased nucleation time. The assembly abnormality was accompanied by the presence of a unique crosslinked tubulin dimer identified by SDS–PAGE analysis. The amount of this crosslinked species roughly correlated with the degree of assembly alteration. These observations support the contention that the crosslinked tubulin species per se confers the property of a decreased nucleation time for microtubule assembly (Boekelheide, 1987b).

Both 2,5-HD-induced testis pyrrole reactivity and testis microtubule assembly alterations were maintained during intoxication. Subsequently, both of these parameters returned to baseline levels. During the recovery period, the loss of abnormal testis microtubule assembly behavior occurred with a half-life of approximately 1–2 weeks. Rat brain tubulin has a half-life for turnover of about 1 week (Forgue and Dahl, 1978), a process regulated by the level of unpolymerized tubulin present in the cell (Caron *et al.*, 1985). If tubulin regulation and turnover occurs by a similar process within the testis, then normal catabolism of tubulin altered by reaction with 2,5-HD can explain the appearance, plateau phenomenon, and resolution of the microtubule assembly abnormality.

We have proposed that altered microtubule assembly in the Sertoli cell is the basic biochemical mechanism underlying 2,5-HD-induced testicular injury (Boekelheide, 1987a). The following sequence of events is hypothesized: (1) intoxication with 2,5-HD results in pyrrole derivatization of Sertoli cell tubulin lysyl ϵ-amines, (2) subsequent pyrrole oxidation results in the production of crosslinked tubulin dimers with altered microtubule nucleation properties, (3) enhanced nucleation changes the number and length of Sertoli cell microtubules, (4) an altered cytoskeleton compromises the sustentacular functions of the Sertoli cell, and (5) a nonsupportive Sertoli cell results in germ cell loss and testicular atrophy. The dynamics of altered testis microtubule assembly elucidated by this time-course study support this proposed mechanism of 2,5-HD-induced testicular injury.

REFERENCES

ANTHONY, D. C., BOEKELHEIDE, K., ANDERSON, C. W., AND GRAHAM, D. G. (1983). The effect of 3,4-dimethyl substitution on the neurotoxicity of 2,5-hexanedione. II. Dimethyl substitution accelerates pyrrole formation and protein crosslinking. *Toxicol. Appl. Pharmacol.* **71,** 372–382.

BOEKELHEIDE, K. (1987a). 2,5-Hexanedione alters microtubule assembly. I. Testicular atrophy, not nervous system toxicity, correlates with enhanced tubulin polymerization. *Toxicol. Appl. Pharmacol.* **88,** 370–382.

BOEKELHEIDE, K. (1987b). 2,5-Hexanedione alters microtubule assembly. II. Enhanced polymerization of crosslinked tubulin. *Toxicol. Appl. Pharmacol.* **88,** 383–396.

BOEKELHEIDE, K. (1988). Rat testis during 2,5-hexanedione intoxication and recovery. I. Dose response and the reversibility of germ cell loss. *Toxicol. Appl. Pharmacol.* **92,** 18–27.

CARON, J. M., JONES, A. L., RALL, L. B., AND KIRSCHNER, M. W. (1985). Autoregulation of tubulin syn-

thesis in enucleated cells. *Nature (London)* **317,** 648–651.

CHAPIN, R. E., MORGAN, K. T., AND BUS, J. S. (1983). The morphogenesis of testicular degeneration induced in rats by orally administered 2,5-hexanedione. *Exp. Mol. Pathol.* **38,** 149–169.

CHAPIN, R. E., NORTON, R. M., POPP, J. A., AND BUS, J. S. (1982). The effects of 2,5-hexanedione on reproductive hormones and testicular enzyme activities in the F-344 rat. *Toxicol. Appl. Pharmacol.* **62,** 262–272.

DECAPRIO, A. P., AND JACKOWSKI, S. J. (1987). Comparative neurotoxicity and pyrrole-forming potential of 2,5-hexanedione and perdeuterio-2,5-hexanedione. *Toxicologist* **7,** 130.

DECAPRIO, A. P., OLAJOS, E. J., AND WEBER, P. (1982). Covalent binding of a neurotoxic *n*-hexane metabolite: Conversion of primary amines to substituted pyrrole adducts by 2,5-hexanedione. *Toxicol. Appl. Pharmacol.* **65,** 440–450.

DECAPRIO, A. P., STROMINGER, N. L., AND WEBER, P. (1983). Neurotoxicity and protein binding of 2,5-hexanedione in the hen. *Toxicol. Appl. Pharmacol.* **68,** 297–307.

FAWCETT, D. W. (1975). Ultrastructure and function of the Sertoli cell. In *Handbook of Physiology. Endocrinology: Male Reproductive System* (R. O. Greep, Ed.), Vol. 5, Sect. 7, pp. 21–55. Williams & Wilkins, Baltimore.

FORGUE, S. T., AND DAHL, J. L. (1978). The turnover rate of tubulin in rat brain. *J. Neurochem.* **31,** 1289–1297.

GENTER, M. B., SZAKAL-QUIN, GY., ANDERSON, C. W., ANTHONY, D. C., AND GRAHAM, D. G. (1987). Evidence that pyrrole formation is a pathogenetic step in γ-diketone neuropathy. *Toxicol. Appl. Pharmacol.* **87,** 351–362.

GILLIES, P. J., NORTON, R. M., BAKER, T. S., AND BUS, J. S. (1981). Altered lipid metabolism in 2,5-hexanedione-induced testicular atrophy and peripheral neuropathy in the rat. *Toxicol. Appl. Pharmacol.* **59,** 293–299.

JOHNSON, K. A., AND BORISY, G. G. (1977). Kinetic analysis of microtubule self-assembly *in vitro*. *J. Mol. Biol.* **117,** 1–31.

PARVINEN, L.-M., SODERSTROM, K.-O., AND PARVINEN, M. (1978). Early effects of vinblastine and vincristine on the rat spermatogenesis: Analyses by a new transillumination-phase contrast microscopic method. *Exp. Pathol. Bd.* **15,** 85–96.

ROSENBERG, C. K., ANTHONY, D. C., SZAKAL-QUIN, GY., GENTER, M. B., AND GRAHAM, D. G. (1987). Hyperbaric oxygen accelerates the neurotoxicity of 2,5-hexanedione. *Toxicol. Appl. Pharmacol.* **87,** 374–379.

RUSSELL, L. D., MALONE, J. P., AND MACCURDY, D. S. (1981). Effect of the microtubule disrupting agents, colchicine and vinblastine, on seminiferous tubule structure in the rat. *Tissue Cell* **13,** 349–367.

SODERSTROM, K.-O., AND ROYTTA, M. (1986). Short-time effects of taxol on the seminiferous epithelium of the rat. *Cell Tissue Res.* **245,** 591–598.

VOGL, A. W., LINCK, R. W., AND DYM, M. (1983). Colchicine-induced changes in the cytoskeleton of the golden-mantled ground squirrel (*Spermophilus lateralis*) Sertoli cells. *Amer. J. Anat.* **168,** 99–108.