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INTERACTIONS OF CADMIUM WITH INTERSTITIAL TISSUE OF THE RAT TESTES

UPTAKE OF CADMIUM BY ISOLATED INTERSTITIAL CELLS

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Abstract—Previous studies have shown that the administration of cadmium causes extensive necrosis of the testes and, eventually, a high incidence of interstitial cell tumors. However, the interactions of cadmium with interstitial cells of the testes have not been well defined. Therefore, this study was designed to assess the uptake of cadmium into this potential target cell of cadmium carcinogenesis. Interstitial cells were prepared by collagenase dispersion of decapsulated Wistar rat testes and separated from seminiferous tubules by unit gravity sedimentation. Such preparations showed a high exclusion rate of trypan blue. The interstitial cell preparations were incubated at 33° with various concentrations of cadmium (1.0 to 100 μM) for periods ranging from 0.5 to 60 min. At the end of the incubation, cellular cadmium was separated from cadmium in the media by centrifugation through an oil layer. Initial experiments showed three distinct phases of cadmium influx into interstitial cells, a primary rapid velocity phase (V_0; 0 to 1.5 min), a second intermediate velocity phase (V_1; 3 to 12 min), and a third low velocity phase (V_2; 15 to 60 min). V_2 appeared to have both influx and efflux components, as efflux experiments indicated an approximate 20% loss of cadmium from 15 to 60 min. The initial phase was found to be nonsaturable and was not decreased by inclusion of potassium cyanide (1.0 mM), *N*-ethylmaleimide (1.0 mM), or zinc (100 μM) in the incubation mixture. However, V_1 was found to be saturable between 50 and 100 μM cadmium and was substantially decreased by the inclusion of potassium cyanide, *N*-ethylmaleimide or zinc during incubation. These data suggest that cadmium is taken up into interstitial cells by a transport system that may normally function in zinc uptake and may possibly constitute carrier mediated or active transport.

Administration of cadmium salts will result in a selectively injurious effect on mammalian testicular tissue. The action of cadmium on the testes was first clearly documented by Parizek and Zahor [1], although in an earlier report concerned with emetic effect of cadmium, a blue discoloration of the testes was noted in cadmium-treated rats [2]. A single injection of cadmium will result in acute testicular necrosis in various species after which the seminiferous tubules are destroyed permanently, although the interstitial tissue in time regenerates [3–7]. Following such necrotizing doses of cadmium, a high incidence of interstitial cell tumors will occur in both rats and mice [7]. The acute effects of cadmium on the rodent testes occur at doses which are nontoxic to other organs [1] and despite the fact that only a very small fraction of administered cadmium (under 0.2%) will localize in this organ [8, 9].

The interstitial cell tumors induced by cadmium resemble microscopically those induced by ligation of the blood vessels of the testes, prompting Gunn *et al.* [10] to term the effects of cadmium a selective "chemical ligation" of the testicular vasculature. However, cadmium-induced tumors, in contrast to interstitial tumors induced by total vascular ligation, are androgenically active [10, 11] and contain histochemically demonstrable 3 β-hydroxysteroid dehydrogenase activity, a key enzyme in steroid biosynthesis [12]. Interestingly, Maekawa and Imamura [13] have shown that intratesticular ovarian grafts from rats subsequently treated with cadmium are invariably healthy in appearance although the surrounding testicular tissue is destroyed. In contrast, ovarian transplants become a mass of fibrous connective or adipose tissue following surgical ligation of the spermatic artery-pampiniform plexus [13]. Hence, the hypothesis that cadmium induces testicular injury due to an ischemic necrosis resembling that of ligation of the testicular artery would seem inadequate as a complete explanation of the mechanism involved, and more subtle effects on testicular tissue could be of importance. In this regard, testicular cadmium appears to be confined to interstitial tissue and will not traverse into seminiferous tubules [9]. The localization of cadmium in the interstitial tissue, the eventual site of cadmium-induced testicular tumor formation, could be of mechanistic significance. However, no detailed study concerning the physiologic/toxicologic interactions of cadmium with interstitial cells exists in the literature. In this regard, interstitial cells are easily isolated [14] and have been widely used in the field of endocrinology [14–17]. Therefore, the general goal of the present study was to examine the interactions of

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cadmium with interstitial cells isolated from rat testes. Specifically, the uptake of cadmium into this potential target cell of cadmium carcinogenesis was studied.

MATERIALS AND METHODS

Male Wistar rats (approximately 250 g; FCRF, Frederick, MD) were used as the source of testes in all experiments. Animals were killed by cervical dislocation at approximately 9:00 a.m. for each experiment.

Interstitial cells were isolated by the method of Dufau and Catt [14] with slight modification. Testes were removed, carefully decapsulated and deveined, and placed into a 50-ml plastic centrifuge tube containing 2.5 ml/testis of calcium-free Krebs–Ringer solution (121 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.2 mM $NaHCO_3$, 11.1 mM glucose equilibrated with 95% O_2/5% CO_2 and pH 7.4) preheated to 33° and containing 1 mg/ml collagenase (Sigma type IA, Sigma Chemical Co., St. Louis, MO). Generally, four testes were used in each preparation. The tube containing the testes was inundated with 95% O_2/5% CO_2, sealed, placed in a horizontal position, and incubated in a Dubnoff metabolic shaker for 35 min at 33° with shaking (90 oscillations/min). At the end of this initial incubation, tubes were opened and 100 μl of 1.41% $CaCl_2$ for every 15 ml of incubation mixture was added. The tubes were again inundated with 95% O_2/5% CO_2, sealed, and incubated for a further 10 min. At the end of this incubation, the tubes were set on end and allowed to stand for 5 min, during which time the interstitial cells were separated from dispersed seminiferous tubules by unit gravity sedimentation. The supernatant fraction was then pipetted off with precautions taken not to disturb the sediment. An equal volume of Krebs–Ringer solution was then added to the sediment and gently mixed by repeated inversion. The sedimentation and removal of supernatant fraction were repeated. The supernatant fractions were then pooled, filtered through nylon mesh, and centrifuged at 120 *g* for 20 min (4°). The resulting pellet was resuspended in 1 ml/testis equivalent of the following incubation buffer (131 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 1.0 mM $CaCl_2$, 10 mM Tris–HCl, 11.1 mM glucose, pH 7.4). Protein was measured by the method of Bradford [18] and adjusted to a final concentration of 1.0 mg/ml. Viability of the interstitial cell preparations was determined by the trypan blue exclusion test [19] and consistently exceeded 90%, while contamination with spermatozoa was minimal.

For influx experiments, interstitial cell suspensions (1.8 ml) in incubation buffer were preincubated for 10 min at 33° before the addition of $CdCl_2$ (containing ^{109}Cd at 2.5 μCi ^{109}Cd/μmole Cd; New England Nuclear, Boston, MA) dissolved in saline (200 μl). The final concentrations of cadmium ranged from 1 to 100 μM. Incubations were generally for 12 min following the addition of cadmium; during this period, 200-μl samples were removed at 0.5, 1.0, 1.5, 3, 6, 9 and 12 min. In one series of experiments using longer incubation times, samples were additionally taken at 15, 30, 45 and 60 min. The cadmium taken up by interstitial cells was then determined by a modification of the method of Stacey and Klaassen [20]. The 200-μl aliquot of incubation mixture was layered over 100 μl of oil (silicone oil, density 1.05 mixed 1:2, v/v, with dioctyl phthalate, density 0.981, both from the Aldrich Chemical Co., Milwaukee, WI) which had been previously layered over 50 μl of 3 N KOH in a 500-μl polyethylene microcentrifuge tube. The samples were then centrifuged for 15 sec at 9400 *g* in a Beckman model 150 microfuge. The uptake of cadmium was then determined by cutting the tubes at the oil interface, after rapid freezing in a dry ice-isopropanol bath, and determining cadmium content of the bottom portion by gamma spectrometry using a Nuclear Chicago model 18 gamma spectrometer. Allowance for adherent fluid was made in the calculation of results. The trypan blue exclusion test indicated that incubation with 100 μM cadmium for 60 min did not result in decreased cellular viability (exclusion rate = 91.6 ± 1.9%, N = 3) in comparison to cells not incubated with cadmium (92.9 ± 1.4%).

In one series of influx experiments, potassium cyanide, *N*-ethylmalemide and zinc acetate were added to cell suspensions at final concentrations of 1 mM, 1 mM and 100 μM respectively. Potassium cyanide was added 15 min prior to cadmium, while *N*-ethylmalemide was added 1 min prior to cadmium. Zinc was added simultaneously with cadmium. The effects of these agents on the influx of cadmium (1.0 μM) were then assessed as above.

For efflux experiments, interstitial cell suspensions (1.8 ml) were preincubated for 10 min at 33° before the addition of 200 μl of 200 μM cadmium (final concentration 20 μM) followed by a further 15-min incubation. At the end of this period, 20 ml of ice-cold incubation buffer was added to the cell suspension, which was then mixed and centrifuged at 200 *g* for 2 min (4°). The supernatant fraction was removed and discarded, and cells were resuspended in 1 ml/testis equivalent of fresh incubation buffer. After a 2-min preincubation at 33°, aliquots (200 μl) were taken at 0, 15, 30, 45 and 60 min, and cadmium remaining in the cell was determined as above.

The pooled testes from two rats were used for each separate isolated interstitial cell preparation, and the data in this report represent the mean of four to six such separate preparations for each point. Data were analyzed by analysis of variance followed by Dunnett's *t*-test [21] with a value of $P < 0.05$ considered significant.

RESULTS

The uptake of cadmium into isolated interstitial cells was found to be biphasic over the first 12 min (Fig. 1). This was observed for a wide range of cadmium concentrations. A rapid (≤1.5 min) initial uptake was apparent, as well as a slower second phase (3–12 min). The velocity of uptake of the first phase (V_0) was determined from uptake measurements at 0.5, 1.0 and 1.5 min, while the velocity of the second uptake phase (V_1) was calculated from uptake measurements at 3, 6, 9, and 12 min.

Figure 2 shows that uptake of cadmium (20 μM at longer time points ≥15 min) displayed a third appar-

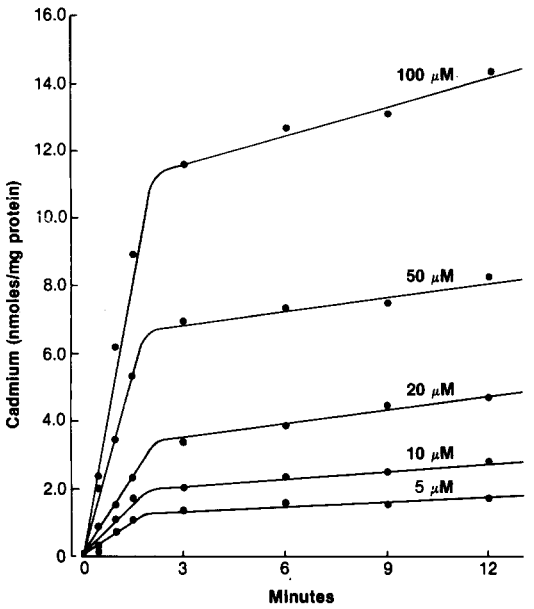

Fig. 1. Uptake of cadmium (5–100 μM) by interstitial cells isolated from rat testes. Each point represents the mean of four to six separate interstitial cell preparations. Concentration of cadmium is indicated above the lines.

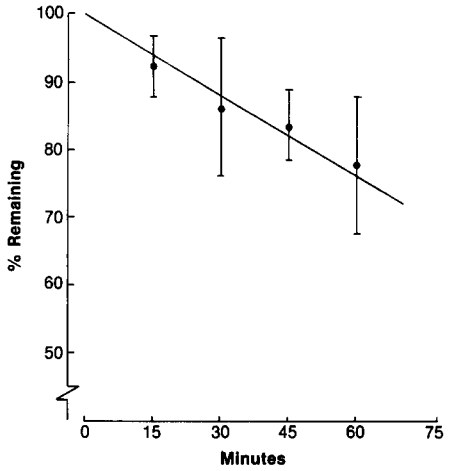

Fig. 3. Efflux of cadmium from interstitial cells isolated from rat testes over 60 min. Isolated cells were preincubated for 20 min in media containing 20 μM cadmium, and switched to fresh media. Efflux was determined at the times indicated. Data represent the mean ± S.E. of four to six separate isolated cell preparations. Time 0 determinations of cadmium content are taken as "100% remaining". Note broken y-axis.

ent phase. The velocity of this third phase (V_2) was less than both the initial and second phases. The possibility that this third phase constituted a mixture of influx and efflux was explored (Fig. 3). A substantial efflux of cadmium out of interstitial cells was detected over a 60-min period.

Figure 4 shows the relationship of the initial velocity (V_0) of cadmium uptake into interstitial cells relative to the cadmium concentration in the incubation mixture (S_0). A linear relation was seen between the incubation mixture concentration of cadmium and the uptake velocity of this phase, at substrate concentrations up to 100 μM, indicating a lack of saturability of this first uptake process.

In contrast, the relationship between cadmium

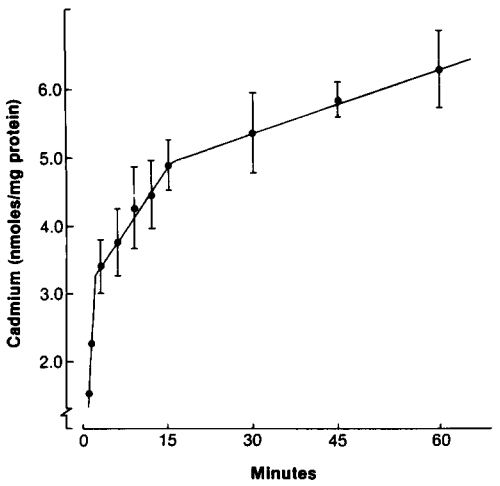

Fig. 2. Uptake of cadmium (20 μM) by interstitial cells isolated from rat testes at times up to and including 60 min. Each point represents the mean ± S.E. of four to six separate interstitial cell preparations. Standard errors have been omitted at the earliest time points for clarity. Note broken y-axis.

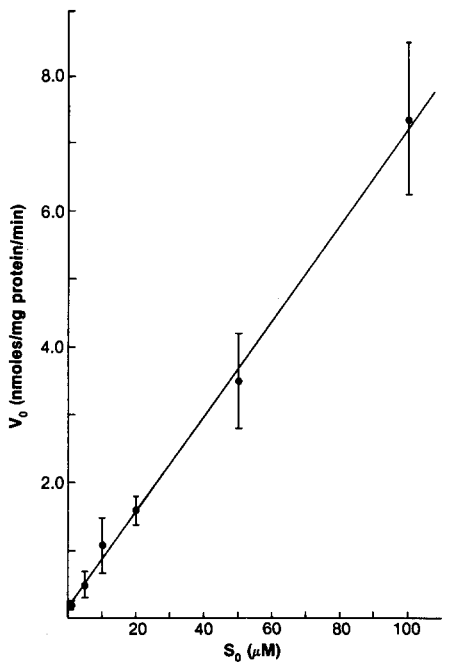

Fig. 4. Initial velocity of cadmium, V_0, into isolated interstitial cells as calculated from 0 to 1.5 min and plotted against the initial concentration of cadmium (S_0) in the incubation media. Data represent the mean ± S.E. of four to six separate interstitial cell preparations. A linear correlation ($r^2 = 0.98$) was seen between V_0 and S_0 at all concentrations used.

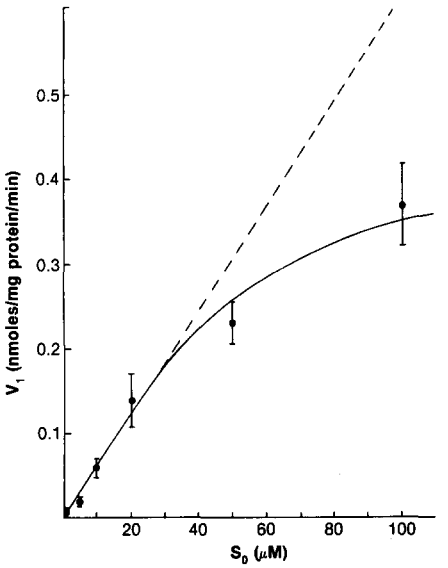

Fig. 5. Velocity of the second phase (V_1) of cadmium uptake into isolated interstitial cells as calculated from 3 to 12 min and plotted against the initial concentration of cadmium (S_0) in the incubation media. Data represent the mean ± S.E. of four to six separate interstitial cell preparations. A linear relationship ($r^2 = 0.99$) was seen between V_0 and S_0 at concentrations of cadmium ≤20 μM, which is representatively continued by the broken lines. At concentrations >20 μM there was a marked deviation from linearity.

concentration and uptake velocity of the second (V_1) phase deviated markedly from linearity at cadmium concentrations of 50 μM and above (Fig. 5). This relationship appeared to indicate a saturable mechanism of uptake for this phase.

The possible inhibitory effects of zinc, potassium cyanide and *N*-ethylmaleimide on the first two phases of cadmium (1.0 μM) uptake into isolated interstitial cells were also investigated. These agents had no effect on the initial velocity (V_0) of cadmium uptake (Fig. 6). However, the second phase of cadmium uptake was reduced significantly by all three agents (Fig. 7). Zinc was the most effective compound in inhibiting cadmium uptake during this phase, while the inhibitor of oxidative metabolism, potassium cyanide, and the sulfhydryl-blocking agent, *N*-ethylmaleimide, also caused substantial decreases in cadmium uptake.

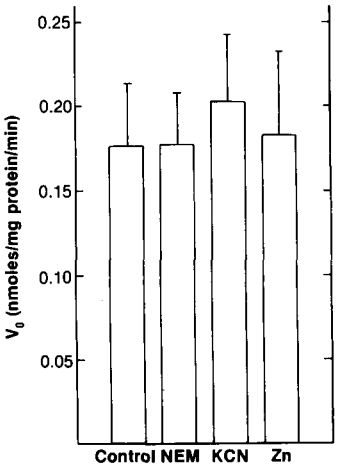

Fig. 6. Effects of *N*-ethylmaleimide (NEM; 1.0 mM), potassium cyanide (KCN; 1.0 mM) and zinc (Zn; 100 μM) on the initial velocity (V_0) of uptake of cadmium (1.0 μM) into isolated interstitial cells. Data represent the mean ± S.E. of four separate isolated cell preparations. KCN was added 15 min prior to cadmium, while NEM and zinc were added 1 min prior and simultaneously with cadmium respectively.

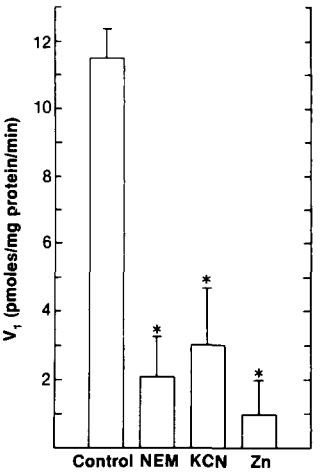

Fig. 7. Effects of *N*-ethylmaleimide (NEM; 1.0 mM), potassium cyanide (KCN; 1.0 mM) and zinc (Zn; 100 μM) on the velocity of uptake of the second phase (V_1) of cadmium uptake into isolated interstitial cells. Data represent the mean + S.E. of four separate isolated cell preparations, and an asterisk (*) represents a significant difference from control $P < 0.05$). Inhibitors of uptake were added as outlined in the legend to Fig. 6.

DISCUSSION

The interstitial cells of the rodent testes may constitute a target cell population for cadmium toxicity, as a single dose of this heavy metal results in the rapid onset of a high incidence of interstitial cell tumors [7, 10, 11]. Despite this observation, little is known about the interactions of cadmium with this apparent target tissue on the cellular level. The use of isolated interstitial cells in the present study allowed a detailed analysis of one important aspect of the interaction of cadmium with these cells, specifically membrane transport. Indeed, it has been emphasized recently that membrane transport could be an important aspect of the metabolism of metals relative to their carcinogenicity [22].

The results of the present study indicate that cadmium is rapidly taken up into isolated interstitial cells in a temporally multiphasic manner over a wide range of cadmium concentrations. The primary phase of this cadmium uptake showed the characteristics typical of passive diffusion. The secondary phase of this accumulation, however, appeared to be saturable (Fig. 5) and was markedly reduced by the inclusion of excess zinc in the incubation media

(Fig. 7). Furthermore, this secondary phase of cadmium uptake also appeared to require reactive sulfhydryl groups and was energy dependent. Similar observations have been obtained with hepatocytes [20, 23, 24], one of the most widely studied cell systems with regard to cadmium transport. The results of Stacey and Klaassen [20] indicate that cadmium uptake into isolated hepatocytes consists of more than one discrete phase, while Failla *et al.* [23] have found that cadmium accumulation in cultured liver parenchymal cells requires reactive sulfhydryl groups. There is also evidence that cadmium uptake into isolated hepatocytes is saturable [24], although this finding has not been observed consistently [20]. Several studies have also indicated an antagonism between zinc and cadmium uptake into liver cells [20, 23, 25], suggesting that, like the results of the present study with interstitial cells, these metals are transferred across the cell membrane by the same mechanism [23, 25].

The velocities of cadmium uptake into interstitial cells noted in this study are similar to those reported for isolated hepatocytes [20]. However, *in vivo* studies have shown that the bulk (approximately 60%) of a dose of cadmium will accumulate in the liver [26] while the testes accumulate much less (<0.2%) [8, 9]. Regional blood flow and organ size would have an obvious effect on this organ distribution, although this would not appear to fully explain such a large difference. The relative proximity of each hepatocyte to the blood supply of the liver, as opposed to that of the interstitial cells of the testes [27], as well as differences in structure of the microvasculature [27, 28] may also, in part, account for the differences observed in the organ distribution of cadmium.

Concurrent, high dose, zinc administration will prevent the necrotic effects and subsequent development of interstitial cell tumors in the rat testes following cadmium exposure [5, 7, 29]. However, the mechanism of this antagonism is not known. Interestingly, the results of Gunn *et al.* [9] indicate that cadmium is confined to the interstitial tissue of mouse testes, and apparently does not traverse the seminiferous tubules. The inhibition of cadmium uptake into interstitial cells suggests a potential mechanism for this zinc antagonism of cadmium-induced tumorigenesis. In this regard, it has been suggested that the prevention of cadmium toxicity in the testes by zinc is related to the concentration of zinc in interstitial tissue [9]. However, further research is required to determine the exact mechanism involved in this zinc-induced amelioration of cadmium toxicity.

In contrast to liver cells, where in certain systems (i.e. perfused liver) efflux of cadmium appears to be insignificant [30], a substantial efflux of cadmium from interstitial cells was detected in the present study. It is thought that the uptake of cadmium into hepatocytes is, in part, dictated by the binding of this metal to intracellular components, including metallothionein [20, 23], a sulfur-rich protein with an extremely high affinity for cadmium [31, 32]. Indeed, where efflux of cadmium from hepatocytes is seen, it ceases coinciding with increasing cadmium binding to intracellular metallothionein [33]. In this regard, recent evidence indicates that metallothionein may be absent in the rat testes [34, 35]. The absence of such a high-affinity intracellular ligand could, in part, account for the efflux of cadmium from interstitial cells detected in the present study.

In conclusion, the results of the present study indicate that cadmium is taken up into isolated interstitial cells in a multiphasic manner, the secondary phase of which was saturable, dependent on reactive sulfhydryl groups, energy dependent, and inhibited by zinc.

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