Journal of Environmental Pathology, Toxicology and Oncology, 19(3):275–280 (2000)

Activation of Androgen Response Element by Cadmium: A Potential Mechanism for a Carcinogenic Effect of Cadmium in the Prostate

*Jianping Ye, Suwei Wang, Mark Barger, Vincent Castranova, and Xianglin Shi**

Cadmium is a transition metal that has been widely used in industry. Epidemiological and animal studies have demonstrated a carcinogenic effect of cadmium on the prostate. Although it has been established that androgen is required for this cancer-inducing process, it is not clear how cadmium interacts with androgen. In this study, the carcinogenic mechanism of cadmium was explored with a focus on interaction of androgen and cadmium at the gene transcription level. An androgen response luciferase reporter was used for analysis of the cadmium activity in the transient transfection assay. Human prostate epithelial cells (LNCap) and liver cells (HepG2) were transfected by the reporter. The result showed that cadmium was able to activate the reporter in the absence of androgen, and that this activation was dependent on the presence of androgen receptor. Cadmium could enhance the androgen response when both androgen and cadmium were applied together to the reporter-transfected cells. Activation of the reporter by cadmium was not associated with cell proliferation or interleukin 6 (IL-6) production, which was proposed to be involved in cadmium-induced carcinogenesis in other experimental systems. Cadmium exhibited a weak ability to induce AP-1. The results demonstrate that cadmium has an androgen-like activity in prostate epithelial cells, and this activity implies a new mechanism for the carcinogenic effect of cadmium in the prostate.

KEY WORDS: cadmium, prostate cancer, androgen, LNCap, HepG2.

Introduction

Cadmium has a broad application in the industry, including battery and cadmium alloy manufacture, electroplating, and metal coating. Approximately 50,000 workers in the United States are occupationally exposed to cadmium.[3] Exposure to cadmium may result in chronic toxicity because of its extremely long biological half-life. At present, there are no proven effective treatments for chronic cadmium toxicity.

Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505. Address all correspondence to Dr. Xianglin Shi, PPRB of NIOSH, 1095 Willowdale Road, Morgantown, WV 26505, xas0@cdc.gov.

Cadmium toxicity is of both occupational and environmental concern. Cadmium was designated as a human carcinogen by the International Agency for Research on Cancer (IARC) in 1993. It is the only metal that has been reported to be capable of inducing tumor in the prostate, which has been demonstrated in animal experiments.[9, 21, 22]

Prostate cancer has received increasing attention in the United States because it is the second leading cause of cancer death.[24] Animal studies have shown that cadmium administrated by various routes was able to induce prostate cancer in rats.[9, 21, 22] Although induction of prostate cancer by cadmium requires input of testicular function,[20, 21] the mechanism by which this metal causes prostate cancer remains to be elucidated. Because it is well established that testosterone

0731-8898/00/$5.00
© 2000 by Begell House, Inc.

(one form of androgen), a male hormone released from the testes, plays a key role in prostate cell proliferation following its conversion to dihydrotestosterone in the prostate, it is possible that cadmium promotes prostate cancer by enhancing androgen activity.

In this study, the interaction between cadmium and androgen was investigated with a focus on transcriptional regulation of the target gene of androgen. The study was carried out using a luciferase reporter plasmid controlled by an androgen response element. The results indicate that cadmium has androgen-like activity and is able to enhance androgen-mediated transcriptional activation. This biological effect of cadmium is dependent on the androgen receptor.

Materials and Methods

Cells and Reagents

The human prostate tumor cell line, LNCap (ATCC, CRL-1740), and human liver tumor cell line, HepG2 (ATCC, HB-8065), were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 supplemented with 5% fetal calf serum, 2 mM glutamine, 2 mM HEPES, and 0.075% Na_2HCO_3 (pH7.4). The cells were cultured in 75 cm^2 cell culture flasks and trypsinized with 0.5% trypsin EDTA when confluence was reached. Cadmium chloride and androgen (dihydrotestosterone) were purchased from Sigma (St. Louis, Missouri).

Transient Transfection Assay

A luciferase reporter plasmid was constructed by inserting the thymidine kinase (TK) promoter upstream of the luciferase gene in the pGL-2 basic plasmid (Promega, Madison, WI). Three copies of androgen response element from the prostate specific antigen gene promoter were linked to the TK promoter at the 5 primer end. The reporter plasmid was amplified in maxiprep and stored at –20 °C. The cells were plated in 6-well culture plates at a density of 1–3 × 10^5/well. The experiment was conducted by transfecting 1 µg of the reporter DNA together with 0.2 µg ß-galactosidase reporter plasmid (internal control) into 3 × 10^5 LNCap cells/well in a 6-well plate. Phenol red free RPMI-1640 and charcoal treated serum were used for preparation of the culture medium. Cadmium chloride was used as a source of cadmium and was added into the transfected cells 24 hours after transfection. Cells were exposed to cadmium for 16 hours. The cells then were harvested for reporter assay. In transfection of HepG2 cells, the cells were plated in a 6-well cell culture plate at a cell density of 1 × 10^5/well. The androgen reporter plasmid (1 µg) was delivered into the cells together with 0.2 µg of ß-galactosidase reporter plasmid (as an internal control) in each well by lipofectamine. Androgen receptor (AR) expression plasmid (0.2 µg) was used to introduce the receptor into the cells. The androgen reporter activity was examined at 48 hours after transfection. The transfection was mediated by lipofectamine reagent (GIBCO BRL, Gaithersburg, MD). A ß-galactosidase reporter plasmid was cotransfected as an internal control to the androgen response reporter. The cells were treated with various concentrations of cadmium chloride, as indicated in the legend to Figure 1, for 24 hours. The cell lysate was used in the reporter assay. Luciferase and ß-galactosidase activity were determined by protocols described previously.[27] The luciferase reporter activity was normalized with the ß-galactosidase activity.

Data Analysis

Data are reported as mean ± SD of 3 individual experiments and were analyzed by Student's *t*-test at a confidence level of $p \leq 0.05$.

Results

Cadmium Activates the Androgen Response Element in LNCap Cells. Androgen receptor is activated by the binding of androgen, and this leads to translocation of the activated receptor from cytoplasm into the nucleus.[13] In the nucleus, the activated androgen receptor recognizes its binding element in target genes and then regulates transcription. Messenger RNA of the target gene will direct protein synthesis. In the current experimental system, androgen-induced response was monitored using a luciferase reporter. The enzymatic activity of the luciferase protein expressed from the reporter gene serves as an indicator of responsiveness of the androgen target gene to androgen or cadmium. The enzymatic activity of luciferase was determined by the intensity of fluorescence emitted from the luciferase substrate luciferin. When androgen was applied, this reporter system exhibited a strong inducible activity. LNCap cells express the androgen receptor.

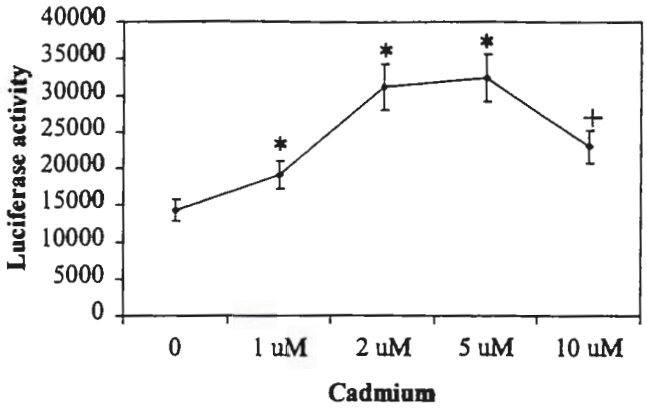

FIGURE 1. Activation of the androgen reporter by cadmium in LNCap cells. LNCap cells were plated in a 6-well culture plate at 3×10^5 cells/well 2 days before transfection. The cells were transfected as stated in the Material and Methods section. Twenty four hours after transfection, cells were treated with various concentration of cadmium ($CdCl_2$) as indicated. The cells were harvested for the reporter assays 16 hrs later. Each point represents a mean ± SD from 3 independent experiments. * indicates a significant increase from the control ($p \leq 0.05$). + indicates a significant decrease from the activity induced by 5 μM cadmium ($p \leq 0.05$).

In this study, the androgen reporter plasmid was introduced into LNCap cells by lipofectamine reagent, and responsiveness of the reporter to cadmium was determined in the transient gene transfection assay. The results show that cadmium was able to activate the reporter activity and the activation was associated with cadmium concentration (Figure 1). A light induction of the reporter activity was observed at 1 μM cadmium, and this activation was maximized at 2–5 μM cadmium. Further increase in cadmium concentration led to a decrease in the reporter responsiveness. These results demonstrate that in the absence of androgen, cadmium was able to activate the androgen responsive reporter. Therefore, it is possible that cadmium may share a biological activity with androgen.

Cadmium Activity is Dependent on the Androgen Receptor. The androgen receptor is a transcription factor, and its activation is controlled by the availability of androgen. The above experimental results demonstrate that cadmium activates the androgen response elements in the reporter plasmid. Because cadmium is not an analog of androgen, it was interesting to speculate on how cadmium activated the reporter.

Two possibilities may exist to explain the above experimental observation: 1) cadmium may act on the reporter in a nonspecific way, and the androgen receptor may not be required for cadmium activity; 2) cadmium may activate the androgen reporter in a specific way, and the specificity is mediated by the androgen receptor. These possibilities were tested by employing an androgen-receptor-negative cell line. If the cadmium activity was observed in the androgen-receptor-negative cells, the first possibility would be correct. Otherwise, the second possibility would be right. In addition, we examined whether the cadmium effect was restricted to prostate epithelial cells. To answer these questions, we used HepG2 cells, a human liver tumor cell line, as a model in which the endogenous androgen receptor is not expressed.

The result showed that cadmium was not able to induce the reporter activity in HepG2 cells that lack AR (Figure 2). This suggests that in the absence of AR, cadmium had no effect on the androgen response element in the reporter. However, in

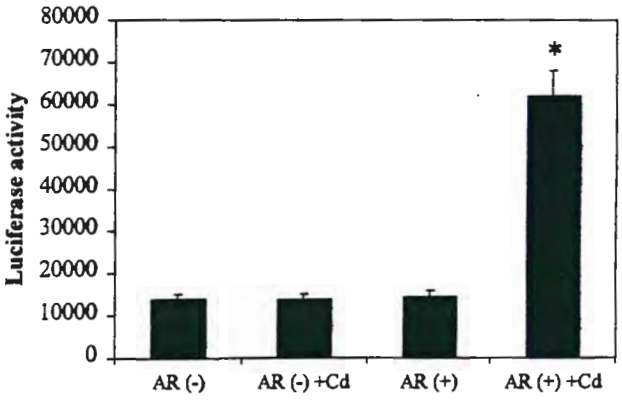

FIGURE 2. Activation of the androgen reporter by cadmium in HepG2 cells. HepG2 cells were plated in a 6-well culture plate at 1×10^5 cells/well 1 day before transfection. The cells were transfected as stated in the Material and Methods section. Twenty four hours after transfection, cells were treated with 2 μM cadmium ($CdCl_2$). The cells were harvested for the reporter assays 24 hrs later. Each point represents a mean ± SD from 3 independent experiments. * indicates a significant increase from the control ($p \leq 0.05$).

the presence of cadmium, expression of AR in HepG2 cells increased the reporter activity approximately fivefold (Figure 2). The androgen receptor alone did not change the reporter activity in the absence of cadmium. These results suggest that the cadmium-induced response is dependent on the androgen receptor. Because both liver cells and prostate epithelial cells responded to cadmium, the cadmium-induced androgen response is not restricted to prostate epithelial cells alone.

Enhancement of Androgen Activity by Cadmium. Because both androgen and cadmium are able to induce androgen reporter activity, we examined the combination effect of cadmium and androgen. Androgen activity was observed with the androgen response reporter. The results show that the androgen-induced response was dose-dependent (Figure 3). An increase in reporter activity was observed at 0.01 nM androgen, and the response became stronger as the androgen dose increased to 10 nM. In the presence of cadmium (2 μM), the androgen effect was enhanced at the following concentrations: 0.01 nM, 0.1 nM, and 1 nM. A higher concentration of cadmium became inhibitory to the androgen activity (data not shown).

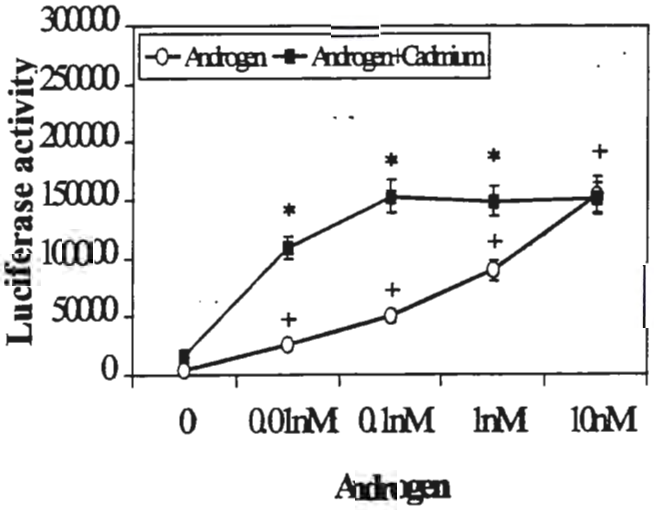

FIGURE 3. Enhancement of androgen reporter activity by cadmium. LNCap cells were transfected under the same condition as stated in the Figure 1. Cadmium (2 μM) was used in combination with different concentration of androgen as indicated in the figure. Each point represents a mean ± SD from 3 independent experiments. + indicates activation of the androgen reporter above control by androgen ($p \leq 0.05$). * indicates a significant increase from the activity induced by androgen alone at each concentration ($p \leq 0.05$).

Discussion

The carcinogenic effect of cadmium on the prostate is dependent on androgen.[20, 21] Testicular androgen is essential for the growth and maintenance of the prostate. Animal studies have demonstrated that exposure to a high dose of cadmium induced degeneration of the testes in the rat because the testis is sensitive to cadmium toxicity.[21] Under this condition, cadmium failed to induce prostate cancer because the prostate epithelial cells cannot grow in the absence of androgen caused by testes damage. This explanation was supported by another animal experiment in which zinc was used to protect the testis against toxicity of cadmium.[20] When zinc is given at doses sufficient to prevent cadmium-induced chronic degeneration in the testes, cadmium's ability to induce prostate cancer was observed. At a low dose of cadmium that did not result in degeneration of testes, cadmium induced proliferative lesions of the rat ventral prostate.[20] All these results indicate that androgen is required for the potential carcinogenic effect of cadmium.

In this study, we observed that cadmium exhibits an androgen like activity at low doses (1–5 μM). The androgen activity was observed using an androgen-specific reporter gene. The reporter gene assay is a specific and sensitive test to study the function of a transcription factor. In this experiment, the reporter gene is turned on after the androgen receptor is activated in the cells. Androgen is the only natural ligand for the androgen receptor. The present study demonstrates that cadmium was able to activate the reporter in both prostate epithelial cells (LNCap cells) (Figure 1) and liver cells (HepG2 cells) in the presence of AR (Figure 2). This suggests that the androgen- like activity of cadmium is not tissue specific. Moreover, the cadmium effect is dependent on the presence of androgen receptor (Figure 2). Physiologically, prostate epithelial cells express a much higher level of androgen receptor than do other cell types. This might be the basis of tissue specificity of cadmium carcinogenesis in the prostate because the higher level of the androgen receptor makes the prostate cells more sensitive to cadmium. Lack of metallothionein, which serves as a critical molecule in detoxification of the toxic metal

ion,[2] in the prostate cells may also contribute to the tissue specificity.[5,6] The result suggests that cadmium might activate androgen receptor through an unidentified mechanism. Because cadmium (2 μM) could enhance androgen-mediated response (Figure 3), the combination of cadmium and androgen will result in a strong stimulation to the prostate cells in vivo.

Several hypotheses have been proposed for the carcinogenic effects of cadmium, including induction of proto-oncogenes,[1, 8, 10, 18, 23] DNA damage,[7, 17] cell proliferation,[8] and reactive oxygen species.[17, 19, 25, 26] In this study, we tested cadmium activity in the activation of proto-oncogene and in the induction of interleukin 6 (IL-6). It has been reported that cadmium was able to activate proto-oncogene c-fos,[10, 18, 23] whose protein product is c-Fos, a component of transcription factor activator protein 1 (AP-1). We used an AP-1 reporter cell line to test cadmium activity on the AP-1 reporter. The reporter cells contain an AP-1 specific reporter gene (luciferase). If c-fos is activated, the reporter cells would express an increased luciferase activity. The results showed that only a onefold induction of the AP-1 reporter activity was observed with cadmium stimulation (data not shown). This effect is weak compared to a typical AP-1 inducer, such as PMA (a carcinogen), which induces the AP-1 reporter activity by at least tenfold. We also tested the mitogenic effect of cadmium on LNCap cells. ^{3}H incorporation was used to monitor cell proliferation in the presence of cadmium. The results indicated that cadmium at 0.1–10 μM did not induce cell proliferation with or without serum starvation (data not shown). This does not support a role of cell proliferation in the activation of androgen reporter. Gene reporter activity always increases with an accelerated cell proliferation. The LNCap cell line used in this study does not proliferate in response to androgen.

IL-6 was proposed as a growth factor for prostate epithelial cells,[4, 14, 16] and it may promote cell growth through interaction with ErbB2, an epidermal growth factor receptor family member.[15] Cadmium has been reported to induce IL-6 in vivo in animal experiments.[11, 12] IL-6 production was tested in this study by ELISA. The results indicate that cadmium did not induce IL-6 production by LNCap cells in cell culture (data not shown). This may exclude IL-6 activity in the androgen effect of cadmium.

In summary, this study explored a new aspect of mechanisms of cadmium-induced carcinogenesis. The results suggest that cadmium may interact with AR and activate AR's transactivation activity. By using an androgen-specific reporter plasmid, we observed that cadmium was able to activate the androgen reporter gene and that the activation was dependent on the presence of androgen receptor. Cadmium was also able to enhance the androgen-induced activation of the reporter. This effect may be important in the mechanism of cadmium-induced prostate cancer.

References

1. Abshire MK, Buzard GS, Shiraishi N, Waalkes MP. Induction of c-myc and c-jun proto-oncogene expression in rat L6 myoblasts by cadmium is inhibited by zinc preinduction of the metallothionein gene. J Toxicol Environ Health 1996; 48:359–77.
2. Cai L, Satoh M, Tohyama C, Cherian MG. Metallothionein in radiation exposure: Its induction and protective role. Toxicology 1999; 132:85–98.
3. Chettle DR, Ellis KJ. Further scientific issues in determining an occupational standard for cadmium [comment]. Am J Ind Med 1992; 22:117–24.
4. Chung TD, Yu JJ, Spiotto MT, Bartkowski M, Simons JW. Characterization of the role of IL-6 in the progression of prostate cancer. Prostate 1999; 38:199–207.
5. Coogan TP, Bare RM, Bjornson EJ, Waalkes MP. Enhanced metallothionein gene expression is associated with protection from cadmium-induced genotoxicity in cultured rat liver cells. J Toxicol Environ Health 1994; 41:233–45.
6. Coogan TP, Shiraishi N, Waalkes MP. Apparent quiescence of the metallothionein gene in the rat ventral prostate: Association with cadmium-induced prostate tumors in rats. Environ Health Perspect 1994; 102 Suppl 3:137–9.
7. Hamilton–Koch W, Snyder RD, Lavelle JM. Metal-induced DNA damage and repair in human diploid fibroblasts and Chinese hamster ovary cells. Chem Biol Int 1986; 59: 17–28.
8. Hechtenberg S, Schafer T, Benters J, Beyersmann D. Effects of cadmium on cellular calcium and proto-oncogene expression. Ann Clin Lab Sci 1996; 26:512–21.
9. Hoffmann L, Putzke HP, Kampehl HJ, Russbult R, Gase P, Simonn C, Erdmann T, Huckstorf C. Carcinogenic effects of cadmium on the prostate of the rat. J Cancer Res Clin Oncol 1985; 109:193–9.

10. Ishikawa T, Igarashi T, Hata K, Fujita T. c-fos induction by heat, arsenite, and cadmium is mediated by a heat shock element in its promoter. Biochem Biophys Res Commun 1999; 254:566–71.
11. Kayama F, Yoshida T, Elwell MR, Luster MI. Cadmium-induced renal damage and proinflammatory cytokines: Possible role of IL-6 in tubular epithelial cell regeneration. Toxicol Appl Pharmacol 1995; 134:26–34.
12. Kayama F, Yoshida T, Elwell MR, Luster MI. Role of tumor necrosis factor-alpha in cadmium-induced hepatotoxicity. Toxicol Appl Pharmacol 1995; 131:224–34.
13. Lindzey J, Kumar MV, Grossman M, Young C, Tindall DJ. Molecular mechanisms of androgen action. Vitam Horm 1994; 49:383–432.
14. Okamoto M, Lee C, Oyasu R. Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells in vitro. Cancer Res 1997; 57:141–6.
15. Qiu Y, Ravi L, Kung HJ. Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells. Nature 1998; 393:83–5.
16. Siegall CB, Schwab G, Nordan RP, Fitzgerald DJ, Pastan I. Expression of the interleukin 6 receptor and interleukin 6 in prostate carcinoma cells. Cancer Res 1990; 50:7786–8.
17. Snyder RD. Role of active oxygen species in metal-induced DNA strand breakage in human diploid fibroblasts. Mutat Res 1988; 193: 237–46.
18. Templeton DM, Wang Z, Miralem T. Cadmium and calcium-dependent c-fos expression in mesangial cells. Toxicol Lett 1998; 95:1–8.
19. Thevenod F, Friedmann JM. Cadmium-mediated oxidative stress in kidney proximal tubule cells induces degradation of Na(+)/K(+)-ATPase through proteasomal and endo/lysosomal proteolytic pathways [In Process Citation]. FASEB J 1999; 13:1751–61.
20. Waalkes MP, Rehm S. Carcinogenicity of oral cadmium in the male Wistar (WF/NCr) rat: Effect of chronic dietary zinc deficiency. Fundam Appl Toxicol 1992; 19:512–20.
21. Waalkes MP, Rehm S, Riggs CW, Bare RM, Devor DE, Poirier LA, Wenk ML, Henneman JR. Cadmium carcinogenesis in male Wistar [Crl:(WI)BR] rats: Dose-response analysis of effects of zinc on tumor induction in the prostate, in the testes, and at the injection site. Cancer Res 1989; 49:4282–8.
22. Waalkes MP, Rehm S, Riggs CW, Bare RM, Devor DE, Poirier LA, Wenk ML, Henneman JR, Balaschak MS. Cadmium carcinogenesis in male Wistar [Crl:(WI)BR] rats: Dose-response analysis of tumor induction in the prostate and testes and at the injection site. Cancer Res 1988; 48:4656–63.
23. Wang Z, Templeton DM. Induction of c-fos proto-oncogene in mesangial cells by cadmium. J Biol Chem 1998; 273:73–9.
24. Wingo PA, Landis S, Ries LA. An adjustment to the 1997 estimate for new prostate cancer cases. Cancer 1997; 80:1810–3.
25. Yang CF, Shen HM, Shen Y, Zhuang ZX, Ong CN. Cadmium-induced oxidative cellular damage in human fetal lung fibroblasts (MRC-5 cells). Environ Health Perspect 1997; 105:712–6.
26. Yang JL, Chao JI, Lin JG. Reactive oxygen species may participate in the mutagenicity and mutational spectrum of cadmium in Chinese hamster ovary-K1 cells. Chem Res Toxicol 1996; 9:1360–7.
27. Ye J, Cippitelli M, Dorman L, Ortaldo JR, Young HA. The nuclear factor YY1 suppresses the human gamma interferon promoter through two mechanisms: Inhibition of AP1 binding and activation of a silencer element. Mol Cell Biol 1996; 16:4744–53.