Effects of Cadmium on Nuclear Protein Kinase C

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Cadmium is a carcinogen whose genotoxicity is only weak. Besides its tumor-initiating capacity, cadmium may be tumor-promoting, since it interferes with several steps of cellular signal transduction. We have investigated effects of cadmium(II) on protein kinase C (PKC), which is a key enzyme in the control of cellular growth and differentiation. Tumor-promoting phorbol esters cause an activation and translocation of PKC from the cytosol to the plasma membrane and to the nucleus of mammalian cells. In mouse 3T3/10 T 1/2 fibroblasts, cadmium(II) potentiated the effect of phorbol ester on nuclear binding and activation of PKC. Furthermore, in a reconstituted system consisting of rat liver nuclei and rat brain PKC, cadmium stimulated the binding of the enzyme to a 105-kDa protein. We propose a model in which cadmium(II) substitutes for zinc(II) in the regulatory domain of PKC, thus rendering the putative protein-protein binding site exposed. Further work is required to elucidate the potential role of the nuclear PKC binding protein(s) in the control of cell proliferation. — Environ Health Perspect 102(Suppl 3):177–180 (1994).

Key words: cadmium, zinc, signal transduction, protein kinase C, nuclear protein kinase C

Introduction

Cadmium is carcinogenic in experimental animals but its genotoxicity is weak. Hence, epigenetic mechanisms have to be considered. In this article we focus on effects of cadmium(II) ions on signal transduction in mammalian cells, which may shift the balance between differentiation and proliferation to the latter state. Cadmium ions have been shown to affect the following levels in cellular signal transduction:

- Signal triggering at the plasma membrane: CdCl₂ at concentrations starting from as low as 0.1 μM total cadmium evokes the inositol trisphosphate signal (1).
- Increase in free intracellular Ca²⁺: CdCl₂ interferes with endoplasmatic reticulum Ca²⁺ ATPase and Ca²⁺ uptake into intracellular vesicles (2).
- Activation and translocation of protein kinase C (PKC): the activating effect of tumor-promoting phorbol esters is enhanced by CdCl₂ (3).
- Activation of oncogene expression: CdCl₂ induces the transcription of the cellular oncogenes c-jun and c-myc in rat myeloblasts at a concentration of 5 μM total cadmium (4).

Since protein phosphorylation is involved on all levels of cellular regulation and protein kinase C is a key enzyme in the control of cellular proliferation, we have to consider metal interactions with this enzyme and especially with its nuclear translocation and activity.

Protein kinase C consists of at least nine isotypes, of which the three major species (α, β, and γ) have closely related structures, each containing a regulatory and a catalytic domain (Figure 1). The conserved region C₅ of the regulatory domain is essential for the binding of the second messenger diacylglycerol or a tumor promoting phorbol ester, both of which induce translocation of the enzyme to cellular structures (5). Furthermore, the conserved region C₇ has two Cys₅His₃ consensus sequences (5), which putatively constitute zinc fingers, since the enzyme contains four zinc atoms in cysteine- and histine-ligated form (6). Additional zinc ions have been shown to enhance the activity and translocation of PKC to the plasma membrane (7,8) or to cooperate with phorbol ester in translocation of the enzyme to the cytoskeleton (9–11). With cadmium ions, activating or inhibiting effects on the activity of PKC have been reported (3). Since we have been interested in the nuclear role of the enzyme, we have studied the modulation of nuclear translocation and activity of PKC by cadmium. Translocation of this enzyme to the cell nucleus has been reported previously (12–15), and endogenous location of PKC in the nucleus has been documented (16,17).

Methods

Protein kinase C was purified from bovine brain as described previously (18). Cell
Table 1. Effects of metal ions on distribution of protein kinase C in mouse fibroblast cell nuclei.8

| Modulators added | Specific activity, pmol 32P/min mg | Total activity, pmol 32P/106 cells |
|------------------|-----------------------------------|-----------------------------------|
|                  | Labile | Fixed | Labile | Fixed |
| Control          | 1041   | 931   | 12.6   | 6.8   |
| TPA, 0.1 µM      | 92     | 1774  | 1.0    | 16.4  |
| A 23187, 1 µM    | 1050   | 1314  | 13.7   | 5.1   |
| Zn2+, 100 µM     | 588    | 940   | 9.9    | 7.9   |
| Cd2+, 50 µM      | 739    | 767   | 13.3   | 4.4   |
| TPA + A 23187    | 127    | 2970  | 2.7    | 18.5  |
| TPA + Zn2+       | 58     | 1842  | 1.4    | 15.0  |
| TPA + Cd2+       | 96     | 4011  | 1.7    | 28.4  |

83T3 10T1/2 mouse fibroblasts were incubated with the modulators at the concentrations indicated in the following time sequences: TPA alone, 20 min; A 23187, 20 min; zinc acetate (combined with 20 µM pyrithione to facilitate uptake), 40 min; cadmium chloride, 60 min. For combined exposures TPA was added 20 min prior to termination of incubation. Data were averaged from three independent experiments, and showed a standard deviation of less than 10%.

culture was done by standard procedures and cell nuclei were isolated according to Malviya et al. (19). Protein kinase C was assayed according to Newton and Koshland (20), phorbol ester binding was analyzed as described by Leach and Blumberg (21), and protein was determined with bicinchoninic acid after Hill and Straka (22). Free metal ion concentrations were adjusted by a metal buffer consisting of 50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, and 2 mM nitrilotriacetic acid, and calculated by a computer program of Fabiato (23) with stability constants from Smith and Martell (24). Metal analysis was performed by electrothermal atomic absorption spectroscopy as described previously (2). SDS polycrylamide gel electrophoresis was done following Lämmli (25). Western blotting was carried out according to Masmoudi et al. (17), and the overlay assay for protein binding of PKC was performed as described by Mochley-Rosen et al. (26). The detailed procedure for the binding assay has been published previously (27).

Results

Effect of Cadmium and Zinc on Nuclear PKC in Mouse Fibroblasts (3T3 10T1/2)

The translocation of PKC from “labile” to “fixed,” chelator-insoluble material of cell nuclei was studied. Labile enzyme is defined as the chelator-soluble fraction, fixed enzyme as chelator-stable, activated particulate fraction. Zinc or cadmium ions alone are not able to induce translocation of PKC to the fixed pool (Table 1). Also, the calcium ionophor A 23187 does not stimulate translocation. However, if the cells were incubated with the phorbol ester TPA, the specific activity of PKC in the fixed pool was increased by a factor of 1.9. If the phorbol ester was combined with the calcium ionophor or with cadmium (50 µM total concentration), the fixed PKC was enhanced by factors of 1.5 or 2.5 over the phorbol ester-induced activity, respectively. It is remarkable that zinc ions did not potentiate the effect of TPA on the nuclear translocation of PKC. The observed potentiation of the effect of TPA by cadmium ions exhibits a time course shown in Figure 2. The translocation of PKC from the labile to the fixed nuclear fraction increases up to 60 min with no further enhancement.

Effect of Cadmium and Zinc on Isolated Protein Kinase C

The potentiation of PKC activation by cadmium observed in mouse fibroblast nuclei could be caused by a modification of the enzyme protein itself or by a modulation of its interaction with nuclear targets. To investigate the former possibility, we used purified PKC from bovine brain and we added Cd2+ and Zn2+ concentrations adjusted by a metal buffer system and calculated as the free ion concentrations. Neither Cd2+ nor Zn2+ at concentrations up to 1 µM free metal ions stimulated the phosphorylating activity or the phorbol ester binding capability of PKC. However, both metals inhibited the kinase activity at concentrations exceeding 1 µM free metal ions (data not shown).

Effects of Cadmium and Zinc on PKC Binding to Nuclear Protein from Rat Liver

Since we did not obtain sufficient amounts of material from cultured cells to characterize the molecules involved in nuclear binding of PKC, we switched to rat liver for further characterization of PKC binding to nuclear proteins. Mochley-Rosen et al. (26) had detected “receptor proteins for activated C kinase,” which could be identified by the binding of phorbodibutyrate (PDBu) to the protein-bound enzyme. We used (H) PDBu to detect the binding of PKC to the protein fraction from rat liver nuclei (Table 2). The nuclear pellet alone did not bind the phorbol ester, hence we could take the amount of PDBu bound as the quantity of the enzyme bound to the nuclear protein fraction. The binding of PKC was enhanced by the addition of 0.1 nM free Cd2+ or 1.0 nM free Zn2+ ions. Figure 3 shows the dependence of this enhancing effect on the concentration of free Cd2+. At the lowest concentration of 10-11 M free Cd2+, 1 µM free Ca2+ cooperated in the effect of Cd2+ on enzyme-protein binding; but over a wide range of Cd2+ concentrations the effect was independent of the presence of a micromolar Ca2+ concentration. A most pertinent issue is, of course, the nature of the nuclear proteins that bind PKC. In a first approach, an “overlay assay” was performed. The nuclear protein fraction was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane strips. These were incubated with purified PKC (Table 3). In the absence of phosphatidylserine and phorbol ester TPA, no binding was detected. In the presence of the cofactors, PKC binding to a 50-kDa protein was detected, which was further intensified when 0.1 nM free Cd2+ was present. In the presence of cofactors and Cd2+, PKC was attached also to a 105-kDa protein. This binding was specific to cadmium, since no binding to this band was detected in the absence of the metal (27).
**Figure 2.** Cadmium potentiomation of phorbol-ester induced increase in fixed nuclear PKC activity. Cells were incubated for the times given with 50 μM cadmium in a serum-free medium. Phorbol ester TPA was added to a final concentration of 100 nM 20 min prior to termination of the incubation. Nuclei were isolated and PKC was assayed as given in Methods. PKC activity extracted by EDTA is termed labile; the activity subsequently extracted by 1% Triton X-100 is termed fixed. With TPA alone in the absence of cadmium the specific activity of labile PKC was 92, that of fixed PKC 1776 pmol P/min/mg.

**Figure 3.** Effect of cadmium on phorbol-ester binding to nuclear pellet. 150 μg of nuclear pellet proteins were incubated with 2 units/ml of partially purified PKC at various free cadmium concentrations either in the absence or in the presence of 1 μM free Ca²⁺. [3H]phorbol dibutyrate at 45 nM was present in the binding assay and specific binding was determined in the presence of 100 μM TPA. Incubation was for 30 min at 37°C. 0, incubation with Ca²⁺ alone; 1, incubation with Ca²⁺ plus 1 μM free Ca²⁺. Reproduced from Block et al. (27) with permission.

**Table 3.** Metal enhanced binding of PKC to a 105-kDa nuclear protein.

| Modulators added | Binding of PKC to a 105-kDa nuclear protein |
|------------------|--------------------------------------------|
| Phosphatidylserine + TPA | - |
| Phosphatidylserine + TPA + Zn²⁺ | + |
| Phosphatidylserine + TPA + Ca²⁺ | + |

*Rat liver nuclear protein was separated by SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose filter strips. These strips were incubated for 1 hr at 37°C with 300 μg/ml phosphatidylserine, 0.1 μM phorbol ester TPA, 4 units/ml of purified bovine brain PKC, and, where indicated, with either 1 nM free Zn²⁺ or 0.1 nM free Ca²⁺. Bound PKC was detected with antibody against a partial sequence of PKC (α-fragment).*

**Discussion**

What mechanism of interaction of PKC with zinc and cadmium ions enhances nuclear targeting? In agreement with other researchers, we assume that the two zinc ions in zinc finger motifs have high affinities to a combination of four liganding amino acid side chains (cysteines and histidines). Makowski and Sunderman have determined the dissociation constants of the zinc-finger protein transcription factor IIIA as 1.0×10⁻⁷ M for the zinc-protein and 2.8×10⁻⁹ M for the cadmium-protein complex (29). Hence, zinc finger sites of the regulatory domain of PKC probably will be occupied with Zn²⁺ permanently in the presence of physiologic zinc concentrations; and we propose a model for metal binding to PKC, in which the two types of zinc finger structures are formed by a Cys₃, and a Cys₃His₃ coordination, respectively (Figure 1). This assignment is in accordance with our analysis of ligands for chelator-stable zinc atoms in PKC. Since in addition to the two Cys₃His₃ sequence motifs there are four more histidines in the same regulatory domain of PKC, two of these histidines are proposed to bind the additional modulatory zinc or cadmium ions. The latter ions could enhance the stimulation by phorbol esters of the binding of PKC to the nuclear target proteins. This proposal is further supported by the presence of basic amino acid clusters in the same sequence of PKC which are related to the basic clusters found in other "nuclear targeting" sequences (30,31).

**Conclusion**

In conclusion, our study suggests that, in addition to genotoxic action, epigenetic mechanisms for the carcinogenicity of cadmium should be taken into account. Cadmium stimulates the nuclear targeting of PKC at the very low concentration of the free ion of 0.1 nM. Possibly, this could activate transcription factors by phosphorylation, which in turn could stimulate the expression of proliferation genes.

**REFERENCES**

1. Smith JB, Dwyer SD, Smith L. Cadmium evokes inositol polyphosphate formation and calcium mobilization. J Biol Chem 256:7115–7118 (1981).
2. Hechtenberg S, Beyersmann D. Inhibition of sarcoplasmic reticulum Ca²⁺-ATPase by cadmium, lead and mercury. Enzyme 45:109–115 (1991).
3. Beyersmann D, Hechtenberg S, Block C, Kircher H. Interactions of cadmium with cellular signal transduction. Metal Comp Environ Life 4:187–192 (1992).
4. Jin P, Ringertz NR. Cadmium induces transcription of proto-oncogenes c-jun and c-myc in rat L6 myoblasts. J Biol Chem 265:14061–14064 (1990).
5. Nishizuka Y. Studies and perspectives of the protein kinase C family for cellular recognition. Cancer 63:1892–1903 (1989).
6. Hubbard SR, Bishop WR, Kirschmeier P, George SJ, Cramer P, Hendrickson WA. Identification and characterization of zinc binding sites in protein kinase C. Science 254:1776–1779 (1991).
7. Murakami K, Whiteley MK, Routenberg A. Regulation of protein kinase C activity by cooperative interaction of Zn²⁺ and Ca²⁺. J Biol Chem 262:13902–13906 (1987).
8. Gaermly P, Szamel M, Resch K, Somogy J. Zinc can increase the activity of protein kinase C and contributes to its binding to plasma membranes in T lymphocytes. J Biol Chem 263:6487–6490 (1988).
9. Zalewski PD, Forbes IJ, Giannakis C, Cowled PA, Beets WH. Synergy between zinc and phorbol ester in translocation of protein
kinase C to cytoskeleton. FEBS Lett 273:131-134 (1990).
10. Forbes IJ, Zalewski PD, Giannakis C. Role for zinc in a cellular response mediated by protein kinase C in human B lymphocytes. Exp Cell Res 195:224-227 (1991).
11. Hedberg K, Birell GB, Hayes GO. Phorbol ester-induced actin cytoskeletal reorganization requires a heavy metal ion. Cell Regul 2:1067-1079 (1991).
12. Halsey DL, Girard PR, Kuo JF, Blackshear PJ. Protein kinase C in fibroblasts. Characteristics of its intracellular location during growth and after exposure to phorbol esters and other mitogens. J Biol Chem 262:2234-2243 (1987).
13. Kiss Z, Deli E, Kuo JF. Temporal changes in intracellular distribution of protein kinase C during differentiation of human leukemia HL 60 cells induced by phorbol ester. FEBS Lett 231:41-66 (1988).
14. Leach KL, Powers EA, Ruff VA, Jaken S, Kaufmann S. Type 3 protein kinase C localization to the nuclear envelope of phorbol ester treated NIH 3T3 cells. J Cell Biol 109:685-695 (1989).
15. Divecha N, Banfc H, Irvine RF. The polyphosphoinositol cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-I) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. EMBO J 10:3207-3214 (1991).
16. Buckley AR, Crowe PD, Russell DH. Rapid activation of protein kinase C in isolated rat liver nuclei by prolactin, a known hepatic nitrogen. Proc Natl Acad Sci USA 85:8649-8653 (1988).
17. Masmoudi A, Labourdette G, Mesel M, Huang FL, Huang KP, Vincendon G, Malviya AN. Protein kinase C is located in rat liver nuclei. Partial purification and biochemical and immunocytochemical characterization. J Biol Chem 264:1172-1179 (1989).
18. Block C, Beyersmann D. Purification of bovine brain protein kinase C employing metal ion dependent properties. In: Cellular Regulation by Protein Phosphorylation (Heilmeyer LMG, ed.). New York:Springer, 1991.
19. Malviya AN, Rogge P, Vincendon G. Stereospecific inositol-1,4,5-

$^{32}$P].triphosphate binding to isolated rat liver nuclei; evidence for inositol trisphosphate receptor-mediated calcium release from the nucleus. Proc Natl Acad Sci USA 87:9270-9274 (1990).
20. Newton AC, Koshland DE. High cooperativity, specificity and multiplicity in the protein kinase C-lipid interaction. J Biol Chem 264:14909-14915 (1989).
21. Leach KL, Blumberg PM. Modulation of protein kinase C activity and $^{[3]H}$ phorbol-12,13-dibutyrate binding by various tumor promoters in mouse brain cytosol. Cancer Res 45:1958-1963 (1985).
22. Hill HD, Straka JG. Protein determination using bichoninonic acid in the presence of sulphydryl reagents. Anal Biochem 170:203-208 (1988).
23. Fabia A. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solution containing multiple metals and ligands. Methods Enzymol 157:378-421 (1988).
24. Smith RM, Martell AE. Critical stability constants, Vol 1. New York:Plenum Press, 1974.
25. Lämmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685 (1970).
26. Mochley-Rosen D, Henrich CJ, Cheever L, Khaner H, Simpson PC. A protein kinase C isozyme is transllocated to cytoskeletal elements on activation. Cell Regul 1:693-706 (1990).
27. Block C, Freyermuth S, Beyersmann D, Malviya AN. A role of cadmium in activating nuclear protein kinase C and the enzyme binding to nuclear protein. J Biol Chem 267:19824-19828 (1992).
28. Quest AFG, Bloomenthal J, Bardes, ESG, Bell RM. The regulatory domain of protein kinase C coordinates four atoms of zinc. J Biol Chem 267:10193-10197 (1992).
29. Makowski GS, Sundeman FW. The interactions of zinc, nickel and cadmium with the Xenopus transcription factor IIIA, assessed by equilibrium dialysis. J Inorg Biochem 48:107-119 (1992).
30. Dingwall C, Laskey RA. Nuclear targeting sequences—a consensus? Trends Biochem Sci 16:478-481 (1991).
31. Malviya AN, Block C. A bipartite nuclear targeting motif in protein kinase C? Trends Biochem Sci 17:176 (1992).