Biotechnol. Bioprocess Eng. 2000, 5: 40-43

Effect of Zinc on Vascular Smooth Muscle Cell Death Mediated by PDTC

Sung-Kwon Moon[1] and Sang-Do Ha[2]*

[1] Univ. of Texas Medical Branch, Div. of Cardiology and Sealy, Center for Molecular Cardiology, Galveston, TX 77550, U.S.A.
[2] Dept. of Industry and Technology, Korea Health Industry Development Institute, Seoul 156-050, Korea

Abstract Pyrrolidinedithiocarbamate (PDTC) and *N*-Acetylcysteine (NAC) are metal and nonmetal-chelating antioxidant which can induce rat and human smooth muscle cell death. When the smooth muscle cells from mouse aorta (MASMC) that we successfully cultured recently was exposed to PDTC and NAC in a normal serum state, the cells were induced to death by these compounds. However, PDTC did not induce the cell death in a serum depleted medium. This data suggests that certain factors in the serum may mediate the cytotoxic effect of PDTC. The metal chelator, Ca-EDTA blocked PDTC-induced cell death, but Cu-, Fe-, and Zn-EDTA did not block the PDTC-induced cell death. This data indicated that copper, iron, and zinc in the serum may lead to the cytotoxic effect of PDTC. Investigation of the intracellular zinc level in PDTC-induced smooth muscle cell death using the zinc probe dye *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide shows that only the muscle-containing layers of the arteries have higher level of zinc. As expected, PDTC increased the intracellular fluorescence level of the zinc. In agreement with these results, the addition of an exogenous metal, zinc, induced the vascular aortic smooth muscle cell death which led to an increased intracellular zinc level. We concluded that PDTC induced mouse aortic smooth muscle cell death required not only zinc level but also intracellular copper and iron level. The mechanism of this antioxidant to induce vascular smooth muscle cell death may provide a new strategy to prevent their proliferation in arteriosclerotic lesions.

Keywords: pyrrolidinedithiocarbamate(PDTC), mouse aorta smooth muscle cells(MASMC), zinc

INTRODUCTION

Pyrrolidinedithiocarbamate (PDTC) and *N*-acetylcysteine (NAC) are low-molecular-weight thiol compounds reported to exert numerous effects in biological systems. Some investigators have shown that antioxidants such as PDTC and NAC prevent apoptosis in lymphocytes [1-3], neurons [4,5], and vascular endothelial cells [6]. While others have suggested induction in a cell death in various systems. Several hypotheses have been proposed for the mechanism of action of PDTC-induced cell death. PDTC induces apoptosis in thymocytes by raising the intracellular level of redox-active copper [7]. An increase in intracellular copper levels by PDTC was shown to oxidize endogenous thiols [8]. Some hypothesis have also been proposed for excessive exposure to extracellular zinc which can damage central neurons cortical cells, bovine endothelial cells [9,10].

* Corresponding author
Tel: +82-2-2194-7405 Fax: +82-2-824-1762
e-mail: sdha@khidi.or.kr

Proliferation of vascular smooth muscle cells is one of the important features of arteriosclerosis [11]. Rao and Berk have shown that hydrogen peroxide stimulates the proliferation of vascular smooth muscle cells but inhibits proliferation of vascular endothelial cells [12]. Tsai *et al.* [13] have shown that metal chelator PDTC and non-metal chelator NAC induce rat and human aortic smooth muscle cells death. But, the mechanism of metal chelator PDTC toxicity on smooth muscle cells has been unclear. Even though copper may mediate PDTC toxicity on cellular systems [7], it is possible to consider that metals other than copper may related to cytotoxic effects of PDTC on vascular smooth muscle cells because zinc only exists in muscle containing layers of the arteries [14]. Recently, we have successfully cultured smooth muscle cells from mouse aorta and we tested the zinc toxicity of PDTC-induced cell death on the smooth muscle cells which are known to be involved in atherosclerosis and restenosis after vascular injury. Therefore, we reports PDTC induced mouse aorta smooth muscle cells death due not only to zinc levels but also to copper and iron levels derived from extracellular sources.

MATERIALS AND METHODS

Cell Culture and Reagents

Mouse aortic smooth muscle cells (MASMC) were harvested from male mouse by enzymatic dissociation according to the modified method of Gunther *et. al.* [15]. MASMC was cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, U.S.A.) and supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY, U.S.A.), penicillin (100 units/mL) and streptomycin (100 μg/mL) (GIBCO, Grand Island, NY, U.S.A.). Cells were passed every 3-5 days and the experiments were performed by five to fifteen passage cells from a primary culture. PDTC and NAC were purchased from Sigma (St. Louis, U.S.A.) and *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide (TSQ) was obtained from Molecular Probes (Eugene, OR, U.S.A.). $ZnCl_2$, Zn-EDTA, Cu-EDTA, Ca-EDTA, and Fe-EDTA were purchased from Fluka (Buchs, Switzerland).

Viability Assay

Subconfluent, exponentially growing MASMC cells in 96 well plates were incubated with PDTC or NAC for the indicated times. Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which is based on the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2-H-tetrazolium by mitochondrial dehydrogenase to a formazan product [16] at 495 nm.

TSQ Staning

The intracellular Zn^{2+} was becoming visualized by TSQ, a membrane permeable Zn^{2+}-chelating dye as described by Frederickson *et al.* [17]. In brief, cultures were washed with HCSS and incubated in HCSS containing 0.01% TSQ. Cultures were observed under fluorescent microscopy with an UV filter (excitation 365 nm, dicheoic 400 nm, barrier 450 nm) after 5 min.

RESULTS AND DISCUSSION

PDTC has been described as a prooxidant of rat and human aortic smooth muscle cells [13]. Recently, we have successfully cultured smooth muscle cells from mouse aorta. We examined whether PDTC induces cell death or not by using primary cultures of MASMC. Viability of mouse vascular smooth muscle cells after treatment with PDTC and NAC are shown in Fig. 1. PDTC-induced MASMC death is shown in a dose-dependent manner from 10 to 50 μM (Fig. 1(a)) on 10% FBS medium based on modified MTT assay of cell viability [15]. PDTC is a metal chelator which conditions a thio-antioxidant. To examine the effect of PDTC on chelation of metals, we treated MASMC with PDTC in serum deprivation. In the serum-depleted state, cell death occurred progressively. Twenty four hours after serum deprivation, the viability of MASMC was reduced by 50% compared to the cell viability on 10% serum state. PDTC of 10 to 50 μM did not affect the extent of MASMC death which was induced by serum deprivation (Fig. 1(a)). Whereas NAC is another thio-containing antioxidant that did not be capable of chelating metals. As expected, NAC had same cytotoxic effect on MASMC viability in 10% serum state and serum deprivation (Fig. 1(b)). We hypothesized that the cytotoxic effect of PDTC in MASMC may be mediated by metals.

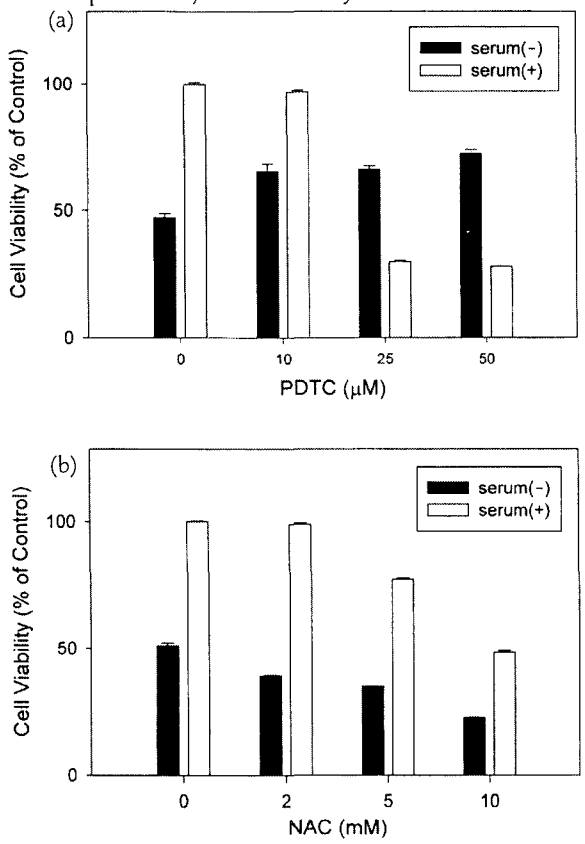

Fig. 1. Viability of mouse vascular smooth muscle cells after treatment with PDTC and NAC; (a) PDTC effect on smooth muscle cells in DMEM with or without 10% FBS, (b) NAC effect on smooth muscle cells in DMEM with or without 10% FBS.

Several studies have reported that PDTC has the ability to chelate metals [7]. Furthermore, recent reports indicate that PDTC induces apoptosis in thymocytes by increasing the intracellular level of copper [7] and bovine cerebral endothelial cell death by increasing the intracellular level of zinc [10].

We next examined the effect of various metal chelators, Ca-, Cu-, Fe- and Zn-EDTA on PDTC induced MASMC death. Fig. 2 indicated that the effects of Cu-, Fe-, Zn-, and Ca-EDTAs on PDTC- induced cell viability. EDTA is a chelator of many divalent and trivalent metals. A metal saturated EDTA can chelate other metals with the release of the metal saturated in it. Calcium-,

Cu-, Fe-, and Zn-EDTA were co-administered with

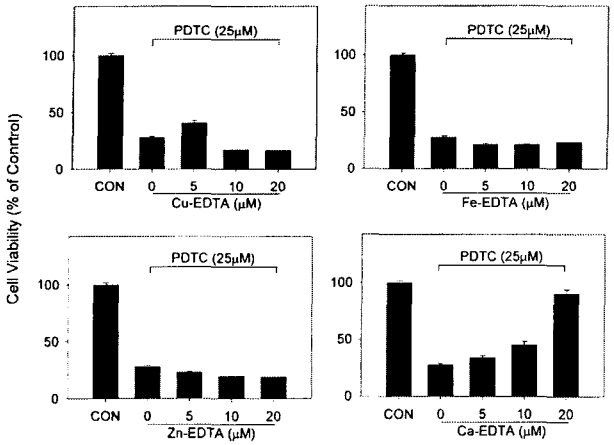

Fig. 2. Effects of Cu-, Fe-, Zn-, and Ca-EDTAs on PDTC-induced cell viability.

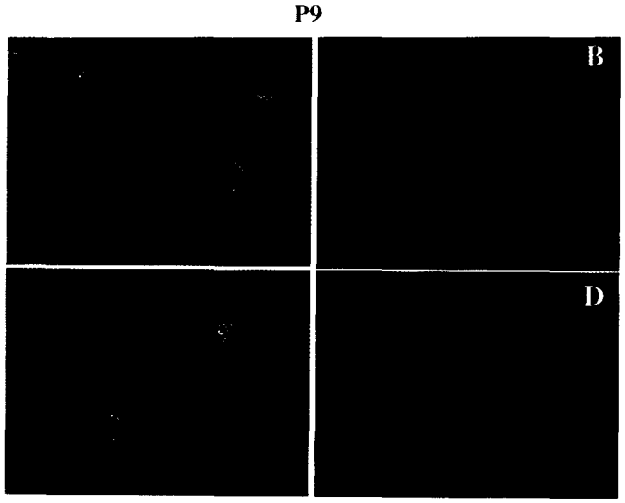

Fig. 3. Effects of PDTC on intracellular TSQ fluorescence staining Bright fields (left panel) and TSQ fluorescence staining (right panel) photomicrographs of vascular smooth muscle cells cultures at 6 hrs following exposure to 25 μM PDTC (c, d) and non treatment (a, b).

PDTC in vascular smooth muscle cells. When cell viability was measured at 24 hr, Zn-, Cu-, and Fe-EDTA did not prevent PDTC-induced cell death (Fig. 2). However, Ca-EDTA blocked the cytotoxic effects at the state of PDTC dose-dependent (Fig. 2). These results show that Cu, Zn, and Fe are the major elements in PDTC-mediated MASMC death.

Spieker *et al.* [14] previously reported that zinc was only detected in the muscle containing layers of the arteries.

Then we measured the changes of intracellular Zinc level using TSQ dye. The effects of PDTC on intracellular TSQ fluorescence staining are shown in Fig. 3. As shown in Fig. 1(a), the viability of MASMC decreased within 6 hr of treatment with PDTC. At this time, The TSQ fluorescence microscopic studies provided an in

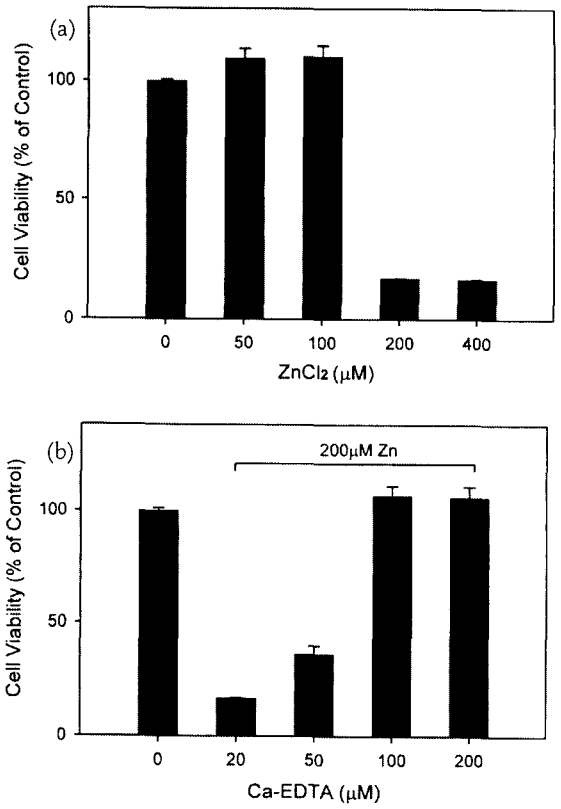

Fig. 4. $ZnCl_2$ cytotoxic effect on vascular smooth muscle cells with and without Ca-EDTA in 10% FBS; (a) Effect of $ZnCl_2$ on vascular smooth muscle cells, (b) Effect of Ca-EDTA on $ZnCl_2$ induced vascular smooth muscle cells death.

creased intracellular zinc staining level (Fig. 3(d)). But, TSQ staining of cultured MASMC showed no zinc staining after 6 h (Fig. 3(b)).

To examine whether extra cellular zinc induces MASMC death or not, we treated $ZnCl_2$ to cultured MASMC. The cytotoxic effect of $ZnCl_2$ on vascular smooth muscle cells with and without Ca-EDTA in 10% FBS are shown in Fig. 4. When $ZnCl_2$ was treated by the cultured MASMC, 200 μM of $ZnCl_2$ induced MASMC death (Fig. 4(a)). However, Ca-EDTA inhibited the cell death induced by $ZnCl_2$ dose-dependently when it was co-administrered with 200 μM of $ZnCl_2$ and Ca-EDTA (Fig. 4(b)). The TSQ staining also showed an increase in the intracellular zinc level in $ZnCl_2$-treated MASMC (data not shown). Consistent with the result of Fig. 4(b), the metal chelator, Ca-EDTA completely quenched the intracellular zinc level induced by $ZnCl_2$ (data not shown).

Arteriosclerosis and its complications such as heart-attack and stroke, are the leading cause of death in the developed countries. Since the proliferation of vascular smooth muscle cells is one of the key features of arteriosclerosis, We found that antioxidant promotes smooth muscle cells death by increased level of metal

ions, especially zinc. These results indicated that this antioxidant treatment may provide as a new therapeutic tool for treatment of arteriosclerosis.

REFERENCE

[1] Hockenbery, D. M., Z. N. Oltvai, X. M. Yin, C. L. Milliman, and S. J. Korsmeyer (1993) Bcl-2 function in antioxidant pathway to prevent apoptosis. *Cell* 75: 241-251.

[2] Roederer, M., F. J. Staal, S. W. Ela, L. A. Herzenberg, and L. A. Herzenberg (1993) *N*-acetylcysteine: potential for AIDS therapy. *Pharmacology* 46: 121-129.

[3] Sandstrom, P. A., M. D. Mannie, and T. M. Buttke (1994) Inhibition of activation-induced death in T cell hybridomas by thiol antioxidants: oxidative stress as a mediator of apoptosis. *J. Leukocyte Biol.* 55: 221-226.

[4] Kane, D. J., T. A. Sarafian, R. Anton, H. Hahn, E. B. Gralla, J. S. Valentine, T. Ord, and D. E. Bredesen (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* 262: 1274-1277.

[5] Rothstein, J. D., L. A. Bristol, B. Hosler, R. H. Jr. Brown, and R. W. Kuncl (1994) Chronic inhibition of superoxide dismutase produces apoptotic death of spinal neurons. *Proc. Natl. Acad. Sci.* U.S.A. 91: 4155-4159.

[6] Abello, P. A., S. A. Fidler, G. B. Bulkley, and T. G. Buchman.(1994) Antioxidants modulate induction of programmed endothelial cell death (apoptosis) by endotoxin. *Arch. Surg.* 129: 134-140.

[7] Nobel, C. S. I., M. Kimland, B. Lind, S. Orrenius, and A. F. G. Slater (1995) Dithiocarbamates induce apoptosis in thymocytes by raising the intracellular level of redox-active copper. *J. Biol. Chem.* 270: 26202-26208.

[8] Burkitt, M. J., H. S. Bishop, L. Milne, S. Y. Tsang, G. J. Provan, C. S. I. Nobel, S. Orrenius, and A. F. G. Slater (1998) Dithiocarbamate toxicity toward thymocytes involves their copper-catalyzed conversion to thiuram disulfides which oxidize glutathione in a redox cycle without the release of reactive oxygen species. *Arch. Biochem. Biophys.* 353: 73-84.

[9] Kim, Y. H., E. Y. Kim, B. J. Gwag, S. Sohn, and J. Y. Koh (1999) Zinc-induced cortical neuronal death with features of apoptosis and necrosis: mediation by free radicals. *Neuroscience.* 89: 175-182.

[10] Kim, C. H., J. H. Kim, J. Xu, C. Y. Hsu, and Y. S. Ahn (1999) Pyrrolidine dithiocarbamate induces bovine cerebral endothelial cell death by increasing the intracellular zinc level. *J. Neurochem.* 72: 1586-1592.

[11] Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362: 801-809.

[12] Rao, G. N. and B. C. Berk (1992) Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ. Res.* 70: 593-599.

[13] Tsai, J. H., M. Jain, D. M. Hsieh, W. S. Lee, M. Yoshizumi, C. Patterson, M. A. Perrella, C. Cooke, H. Wang, E. Haber, R. Schlegel, and M. E. Lee (1996) Induction of apoptosis by pyrrolidinedithiocarbamate and *n*-acetylcysteine in vascular smooth muscle cells. *J. Biol. Chem.* 271: 3667-3670.

[14] Spieker, C., D. Heck, W. Zidek, T. Stratmann, D. P. von Bassewitz, H. Losse, H. Vetter, and H. Zumkley (1986) Elemental and trace element distribution in medical samples: analysis by proton-induced X-ray emission. *Methods Find. Exp. Clin. Pharmacol.* 8(6): 363-366.

[15] Gunter, S., R. W. Alexander, W. J. Atkinson, and M. A. Jr. Gimbrone (1982) Functional angiotensin II receptors in cultured vascular smooth muscle cells. *J. Cell Biol.* 92: 289-298.

[16] Buttke, T. M., J. A. McCubrey, and T. C. Owen (1993) Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. *J. Immunol. Methods* 157: 233-240.

[17] Frederickson, C. J., M. D. Hernandez, and J. F. McGinty (1989) Translocation of zinc may contribute to seizure-induced death of neurons. *Brain Res.* 480: 317-321.

[Received October 28, 1999; accepted December 30, 1999]