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Original Contribution

Vitamin D sensitizes breast cancer cells to the action of H_2O_2: Mitochondria as a convergence point in the death pathway

Gregory E. Weitsman[a,b], Ruth Koren[a,b], Efrat Zuck[a,b], Carmela Rotem[a], Uri A. Liberman[a,b], Amiram Ravid[a,c,*]

[a]*Basil and Gerald Felsenstein Medical Research Center, Sackler Faculty of Medicine, Tel Aviv University, Beilinson Campus, Petah Tikva 49100, Israel*
[b]*Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel*
[c]*Department of Cellular and Developmental Biology, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel*

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Abstract

Calcitriol, the hormonal form of vitamin D_3, sensitizes breast cancer cells to reactive oxygen species (ROS)-dependent cytotoxicity induced by various anticancer modalities. This effect could be due to increased generation of ROS and/ or to increased sensitivity of the target cells to ROS. This work examined the effect of calcitriol on the damage inflicted on breast cancer cells by the direct action of ROS represented by H_2O_2. Treatment of MCF-7 cells with H_2O_2 resulted in activation of caspase 7 as well as induction of caspase-independent cell death. Both were enhanced by 48–72 h of pretreatment with calcitriol. This effect was not due to modulation of H_2O_2 degradation or to a specific effect on $^{\bullet}OH$-mediated cytotoxicity. The H_2O_2-induced drop in mitochondrial membrane potential and release of cytochrome *c* were enhanced by calcitriol. These findings indicate that calcitriol sensitizes breast cancer cells to ROS-induced death by affecting event(s) common to both caspase-dependent and -independent modes of cell death upstream to mitochondrial damage.

Keywords: Calcitriol; Oxidative stress; Programmed cell death; Caspase; Mitochondria; Mitochondrial membrane potential

Introduction

Reactive oxygen species (ROS) are the main cytotoxic mediators of various anticancer modalities such as radiotherapy, photodynamic therapy, and various anticancer drugs. ROS also constitute an important element in the anticancer activities of immune cells, cytotoxic cytokines, and hyperthermia [1]. Superoxide anions, formed in the course of action of many cytotoxic agents, dismutate to yield the long-lived H_2O_2, which in turn undergoes the Fenton reaction to generate highly toxic $^{\bullet}OH$ radicals [2]. Exposure to H_2O_2 may bring about both programmed cell death (PCD) and accidental cell death, the mode of cell death being dependent upon the severity of the oxidative insult. PCD may take the form of apoptosis or necrotic-like cell death depending on the nature of the death pathways

Abbreviations: 3-ABA, 3-aminobenzamide; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CV, crystal violet; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; DNP, 2,4-dinitrophenol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; NAD, nicotinamide adenine dinucleotide; NR, neutral red; PARP, poly(ADP-ribose)polymerase; PCD, programmed cell death; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; zD-DCB, z-Asp-2,6-dichlorobenzoyloxymethylketone.

* Corresponding author. Felsenstein Medical Research Center, Beilinson Campus, Rabin Medical Center, Petah Tikva 49100, Israel. Fax: +1 972 3 9211478.
E-mail address: aravid@post.tau.ac.il (A. Ravid).

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that are activated [3]. ROS were implicated in the cytotoxic action of various agents that a priori are not assumed to cause oxidative stress, e.g., Fas-ligand [4], corticosteroids [5], and growth factor deprivation [6]. It has been shown that both caspase-dependent and -independent modes of cell death may depend on ROS generation. ROS bring about cell death by activation of death pathways and/or by damaging key cellular components such as DNA, mitochondria, and lysosomes [1]. Whereas $O_2^{\bullet -}$ and H_2O_2 cause injury to various cellular constituents, it is the highly reactive $^{\bullet}OH$ radicals that are responsible for most indiscriminant damage. Upon DNA damage the DNA repair enzyme poly(ADP-ribose)polymerase (PARP) ADP-ribosylates nuclear proteins using NAD^+ as a substrate. When DNA damage is extensive, overactivation of PARP consumes NAD^+ leading to ATP depletion, culminating in cell dysfunction and death [7]. PARP is considered one of the mediators of ROS-induced cell death.

The mitochondrion is now a recognized key element in programmed cell death, being an integrator of death and survival signals and a central component of the execution machinery. Mitochondria are well-established targets for ROS action and sites of ROS generation [8]. Both these features play a role in the action of different cytotoxic agents. Stressed mitochondria release into the cytosol a battery of PCD mediators such as cytochrome *c*, Omi/HtrA2, AIF, and Endo G [9]. This process is associated with perturbation of mitochondrial metabolism reflected in a fall of mitochondrial membrane potential that may in turn lead to excessive ROS generation [10].

Accumulating evidence from a variety of epidemiological and experimental studies attests to the in vivo anticancer activity of vitamin D. These studies indicate that endogenously produced or pharmacologically administered hormonally active vitamin D derivatives may lower the incidence and inhibit the progression of various tumors [11–13]. The in vivo anticancer activity of calcitriol may be related to its direct cytostatic and cytotoxic effects on cancer cells [11]. In addition, calcitriol may exert some of its anticancer activity by cooperating with endogenous and therapeutic anticancer agents. Indeed, we and others found that calcitriol increased the susceptibility of cancer cells to the cytotoxic/cytostatic action of tumor necrosis factor α (TNF-α) [14,15], doxorubicin, menadione [16], and ionizing radiation [17,18]. A feature shared by these agents is their ability to bring about excessive ROS generation in their target cells. Moreover, our findings that the interaction between calcitriol and doxorubicin or immune cytokines (TNF-α, interleukins 1 and 6) is markedly inhibited by the addition of antioxidants [16,19,20] are in agreement with the idea that the cross talk between the hormone and these agents is a ROS-dependent process.

This work aims to examine the various facets of the response of breast cancer cells to the common mediator of ROS injury, H_2O_2, and their possible modulation by vitamin D_3.

Materials and methods

Materials

Tissue culture media were purchased from Biological Industries (Beit Haemek, Israel). Tissue culture dishes were from Corning Glass Work (Corning, NY), except for clear-bottom black 96-well microtiter plates obtained from Greiner Labortechnik (Kremsmuenster, Germany). Calcitriol was obtained from Hoffman-LaRoche Co. (Nutley, NJ; a generous gift from Dr. M. Uskokovic). H_2O_2 "Baker analyzed" was from J.T. Baker (Philipsburg, NJ). 3-Amino-1,2,4-triazole (aminotriazole), mercaptosuccinic acid, dimethyl sulfoxide (DMSO), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 3-aminobenzamide (3-ABA), crystal violet (CV), and neutral red (NR) were purchased from Sigma Chemical Co. (St. Louis, MO). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (ac-DEVD-AMC), and z-Asp-2,6-dichlorobenzoyloxy-methylketone (zD-DCB) were from Alexis Biochemicals (Lausen, Switzerland). Monoclonal antibodies to cytochrome *c* and the C-terminus of poly(ADP-ribose) polymerase were from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies directed against the cleaved and intact caspase-7 were from Cell Signaling Technology, Inc. (Beverly, MA). The antibody against mitochondrial voltage-dependent anion channel (VDAC) was from Calbiochem, Inc. (San Diego, CA). Peroxidase-conjugated secondary antibodies, goat anti-rabbit IgG and goat anti-mouse IgG, were from Sigma Chemical Co. and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA), respectively. All other reagents were of analytical grade.

Cell culture

MCF-7 human breast cancer cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/liter glucose and supplemented with 10% fetal calf serum and antibiotics. Cells were subcultured twice weekly. Various numbers of cells were plated as follows: for cytotoxicity assays, 6×10^3 cells/well in 96-well microtiter plates; for H_2O_2 degradation assays 2.5×10^5 cells/60 mm petri dish; for polyacrylamide gel electrophoresis (PAGE), 4.5×10^5 cells/60 mm petri dish; for caspase activity assay, 6×10^5 cells/60 mm petri dish; for mitochondrial membrane potential measurement and microscopy, 5×10^3 cells /well in 96-well black clear-bottom microtiter plates. Cultures were treated with calcitriol 24 h after seeding. The vehicle ethanol was added to control cultures and its concentration never exceeded 0.06%.

H_2O_2 cytotoxicity

MCF-7 cells were incubated in 96-well microtiter plates with calcitriol or vehicle for 72 h (unless otherwise stated).