Vitamin D Is a Prooxidant in Breast Cancer Cells

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ABSTRACT

The anticancer activity of the hormonal form of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)\(_2\)D], is associated with inhibition of cell cycle progression, induction of differentiation, and apoptosis. In addition, 1,25(OH)\(_2\)D\(_3\) augments the activity of anticancer agents that induce ex- cessive reactive oxygen species generation in their target cells. This study aimed to find out whether 1,25(OH)\(_2\)D\(_3\) acting as a single agent, is a prooxidant in cancer cells. The ratio between oxidized and reduced glutathione and the oxidation-dependent inactivation of glyceroldehyde-3-phosphate dehydrogenase (GAPDH) are considered independent markers of cellular reactive oxygen species homeostasis and redox state. Treatment of MCF-7 breast cancer cells with 1,25(OH)\(_2\)D\(_3\) (10–100 nM for 24–48 h) brought about a maximal increase of 41 ± 13% (mean ± SE) in the oxidized/reduced glutathione ratio without affecting total glutathione levels. The in situ activity of glutathione peroxidase and catalase were not affected by 1,25(OH)\(_2\)D\(_3\), as assessed by the rate of H\(_2\)O\(_2\) degradation by MCF-7 cell cultures. Neither did treatment with 1,25(OH)\(_2\)D\(_3\) affect the levels of glutathione reductase or glutathione S-transferase as assayed in cell extracts. The hormone did not affect overall glutathione consumption and efflux as reflected in the rate of decline of total cellular glutathione after inhibition of its synthesis by buthionine sulfoximine. The extent of reversible oxidation-dependent inactivation of GAPDH in situ was determined by comparing the enzyme activity before and after reduction of cell extracts with DTT. The oxidized fraction was 0.13 ± 0.02 of total GAPDH in control cultures and increased by 56 ± 5.3% after treatment with 1,25(OH)\(_2\)D\(_3\), which did not affect the total reduced enzyme activity. Treatment with 1,25(OH)\(_2\)D\(_3\) resulted in a ~40% increase in glucose-6-phosphate dehydrogenase, the rate-limiting enzyme in the generation of NADPH. This enzyme is induced in response to various modes of oxidative challenge in mammalian cells. Taken together, these findings indicate that 1,25(OH)\(_2\)D\(_3\) causes an increase in the overall cellular redox potential that could translate into modulation of redox-sensitive enzymes and transcription factors that regulate cell cycle progression, differentiation, and apoptosis.

INTRODUCTION

Various lines of evidence, supported by pharmacological studies in animal models and a number of epidemiological studies, indicate that the hormonal form of vitamin D, 1,25(OH)\(_2\)D\(_3\), acts as an anticancer agent in vivo (1). Vitamin D is biotransformed by two consecutive hydroxylation steps in the liver and kidney to 1,25(OH)\(_2\)D\(_3\), the hormone responsible for calcium and phosphate homeostasis. It is now recognized that 1,25(OH)\(_2\)D\(_3\) also acts in a paracrine manner and is produced extraregularly by various cells including activated macrophages and some tumor cells (2–4). Such local production may result in accumulation of the hormone within the tumor milieu to levels that exceed those in the circulation.

The in vivo anticancer activity of 1,25(OH)\(_2\)D\(_3\) has been commonly attributed to its direct cytostatic and cytotoxic effects on cancer cells (1). In addition, 1,25(OH)\(_2\)D\(_3\) may exert some of its activity by cooperating with other anticancer agents. We and others have found that 1,25(OH)\(_2\)D\(_3\) and its synthetic analogues increased the susceptibility of cancer cells to the cytotoxic/cytostatic actions of tumor necrosis factor (5–7), interleukin 1, interleukin 6 (8), doxorubicin, menadione (9), and radiation (10). A feature shared by the agents whose potency is increased by 1,25(OH)\(_2\)D\(_3\) is their ability to bring about excessive ROS generation in their target cells (11–13). This common feature suggests the involvement of ROS in the interaction between 1,25(OH)\(_2\)D\(_3\) and these agents. Indeed, we found that the potentiation of the cytotoxic/cytostatic action of doxorubicin or the immune cytokines by 1,25(OH)\(_2\)D\(_3\) is markedly inhibited by the addition of the thiol antioxidant, N-acetylcysteine (8, 9). Moreover, the oxidative stress experienced by cells exposed to TNF, reflected in the reduction in soluble thiol levels late in the death process, was exacerbated when in the presence of 1,25(OH)\(_2\)D\(_3\) (8). Cells are continuously exposed to ROS produced intracellularly by normal aerobic metabolism. It is commonly accepted that, under normal conditions, the cellular level of ROS is tightly controlled and may even serve a physiological function (14, 15). It seems that ROS can also function in signal transduction in mammalian cells, and that the activity of several transcription factors is regulated by the cellular redox state (16–18).

If, indeed, 1,25(OH)\(_2\)D\(_3\) increases the damaging effect of ROS produced in excess, it is possible that it will also enhance the oxidative impact of ROS produced in the course of normal aerobic metabolism. Such an effect may result in perturbation of the cellular redox state and in modulation of the activity of redox-sensitive proteins. Modulation of key redox-sensitive proteins is, in turn, likely to affect signal transduction networks and transcriptional activity (14–18) and thus may contribute to the cytostatic and cytotoxic actions of the hormone on cancer cells. We addressed this possibility by studying the effect of 1,25(OH)\(_2\)D\(_3\) on the glutathione redox state in MCF-7 human breast cancer cells and on the redox-sensitive enzyme GAPDH. The rationale for this approach derives from several well-accepted notions: (a) thiols, and particularly glutathione, provide one of the major cellular protective mechanisms against ROS, and an increase in the ratio between the disulfide oxidation product (GSSG) of glutathione and its reduced form (GSH) is a useful marker of cell oxidative stress (19); (b) the glutathione pair is coupled to other major redox pairs within the cell, and changes in the ratio between GSSG and GSH reflect changes in the overall cellular redox state; and (c) the activity of the glycolytic enzyme GAPDH is extremely sensitive to thiol oxidation because of an essential cysteine residue in its active site. The extent of this oxidation is considered a sensitive marker of cellular ROS homeostasis (20–23).

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). 1,25(OH)\(_2\)D\(_3\) was obtained from Hoffmann-LaRoche Co.
The effect of 1,25(OH)₂D₃ on glutathione homeostasis in MCF-7 human breast cancer cells is shown in Table 1. Treatment with 1,25(OH)₂D₃ (10 or 100 nm) for 24 or 48 h brought about a highly significant increase in the ratio of GSSG:GSH (P = 3.6 × 10⁻⁵), by ANOVA of the results from four independent experiments presented in Table 1 in which 3–4 replicate cultures were treated with 100 nm 1,25(OH)₂D₃ for 48 h. The average increase in the GSSG:GSH ratio in these experiments was 41 ± 13% (mean ± SE); that is equivalent to an increase of 4.3 ± 1.1 mV in the redox potential of glutathione. The increase in the GSSG:GSH ratio is attributable to both a slight, but significant, reduction in GSH levels (P = 0.05; ANOVA) and a marked, significant increase in GSSG (P = 0.004). There was no detectable change in the cellular content of total glutathione.

The effect of the hormone was time-dependent, apparent after 24 h of treatment, but more pronounced after 48 h (experiment 4). Such a pattern is consistent with the genomic mode of action of the vitamin D receptor. The results of experiment 3 indicate that the effect is dose-dependent and already significant at a concentration of 10 nm 1,25(OH)₂D₃. For the sake of comparison, cultures of MCF-7 cells, prepared as described in Table 1, were exposed for 1 h to H₂O₂ (1 mM) and the GSSG:GSH ratio was determined. The average increase in the glutathione redox potential in four independent experiments was 4.7 ± 1.1 mV (mean ± SE). It is noteworthy that the increase in redox potential after chronic exposure to 1,25(OH)₂D₃ was comparable with that after an acute exposure to a cytotoxic concentration of H₂O₂.

The increase in the GSSG:GSH ratio could be attributable to two conceptually different mechanisms. One possibility is that 1,25(OH)₂D₃ modulates intracellular ROS production, and/or ROS handling, and thus increases the oxidative capacity of ROS produced in the course of normal aerobic metabolism. In that case, the change in glutathione redox potential can be considered a reflection of an overall change in the cellular redox state. Another possibility is that the change in the glutathione redox state is attributable to a direct and primary effect of 1,25(OH)₂D₃ on the activity of the enzymatic machinery responsible for glutathione homeostasis.

We first addressed this question by assessing the effect of 1,25(OH)₂D₃ on the in situ activity of the major enzyme responsible for glutathione oxidation, GPx. This was done by assaying the rate of GPx-dependent degradation by MCF-7 cell cultures of exogenously added H₂O₂. As described previously (29), the degradation of H₂O₂ followed first-order kinetics for at least 60 min. The relative contribution of catalase and GPx to the degradation of H₂O₂ was determined by the use of their specific inhibitors, aminotriazole (31) and...
1,25(OH)2 D3 affects neither the level of GPx nor the overall capacity of catalase using the reducing power of NADPH. Our results imply that replenishment depends on the recycling of GSSG by glutathione reductase; this is continuously consumed during the degradation of H2O2, and its transferase activity causes GSH depletion (34). Table 4 shows no detectable effect of 1,25(OH)2D3 on glutathione transferase activity in MCF-7 cell extracts.

Modulation of glutathione consumption and efflux could also affect the glutathione redox state. Fig. 1A shows the effect of a 48-h exposure to the inhibitor of glutathione synthesis, BSO, on total glutathione levels in MCF-7 cell cultures. In accordance with previous reports in various experimental systems (35), exposure to BSO brings about a ~90% decrease in cellular glutathione levels. This effect is

mercaptosuccinate (32), respectively. The inhibitors were used at saturating concentrations as determined in preliminary experiments and were added to the cultures 1 h before and during exposure to H2O2. Table 2 gives the first-order rate constants for H2O2 degradation as obtained by regression analysis of the exponential decay curves. The rate of degradation in the presence of both inhibitors was indistinguishable from the spontaneous degradation of H2O2 in the absence of cells. It may thus be inferred that the combined activity of catalase and GPX fully accounts for the degradation of H2O2 by MCF-7 cells. Therefore, the degradation in the presence of aminotriazole provides a faithful measure to the in situ activity of GPX. Table 3 illustrates that 72-h treatment with 1,25(OH)2D3 (100 nm) had no effect on H2O2 degradation in the presence of aminotriazole. These results imply that the capacity of GPX is unaffected by the hormone. The same is true also for catalase, the other enzyme responsible for H2O2 degradation. The in situ activity of GPX depends on both the enzyme level and on the concentration of GSH. GSH is continuously consumed during the degradation of H2O2, and its replenishment depends on the recycling of GSSG by glutathione reductase using the reducing power of NADPH. Our results imply that 1,25(OH)2D3 affects neither the level of GPX nor the overall capacity of the GSH regenerating system.

These conclusions were reinforced by directly assaying the activity of glutathione reductase and G6PD. The latter is the key and rate-limiting enzyme in the pentose phosphate pathway that is responsible for the generation of NADPH. Enzyme activities were assayed in cell extracts with saturating concentrations of substrates and cofactors. The results in Table 4 show that the same treatment that increased the GSSG-GSH ratio [48 h exposure to 1,25(OH)2D3 (100 nm)] did not affect the activity of glutathione reductase. We did, however, in agreement with a previous report (33), find a significant increase in the activity of G6PD in extracts of 1,25(OH)2D3-treated cells. Another glutathione-dependent enzyme that can affect the GSSG-GSH ratio is glutathione transferase. The peroxidase activity of this enzyme against organic peroxides contributes to the generation of GSSG, and its transferase activity causes GSH depletion (34). Table 4 shows no detectable effect of 1,25(OH)2D3 on glutathione transferase activity in MCF-7 cell extracts.
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dose-dependent and saturates at a concentration of 20 μM (Fig. 1A). 1,25(OH)2D3-treated and untreated cells were exposed to this saturating concentration of BSO and the decline in glutathione levels was monitored as a function of time (Fig. 1B). It is evident that the rate of decline in glutathione is not affected by 1,25(OH)2D3. Assuming that the decline in the cellular glutathione level is the result of glutathione consumption and efflux, this finding indicates that these processes are not affected by the hormone.

Taken together, the results of Tables 3 and 4 and Fig. 1 lead to the conclusion that treatment with 1,25(OH)2D3 does not have a direct effect on the capacity of the major enzymatic systems responsible for glutathione homeostasis. It is thus possible that the change in the GSSG:GSH ratio reflects an overall increase in the cellular redox potential. We further assessed this notion by examining the effect of the hormone on the redox-sensitive enzyme, GAPDH. This household enzyme, known for its role in glycolysis, has an essential cysteine residue in its active site that is extremely sensitive to thiol oxidation (20, 22, 36, 37). Our first objective was to determine the fraction of cellular GAPDH that is oxidized under normal culture conditions. To this end we assayed the activity of GAPDH immediately after the preparation of cell extracts and after a 60-min incubation at 0°C with DTT (40 mM). (Preliminary experiments indicated that there was no difference in total GAPDH activity (assayed after reduction in situ experiments) represents the overall activity of the cultured cells depends on the combined and coordinated activities of GPx and glutathione reductase, which in turn depend upon the availability of GSH and NADPH, respectively. A simple stoichiometric calculation shows that the amount of GSH consumed during the degradation of H2O2 in these experiments (Tables 2 and 3) equals ~14-fold the total glutathione pool (Table 1). Because 1,25(OH)2D3 did not decrease the ability of the GSH regenerating systems to cope with such severe oxidative challenge, it seems safe to conclude that the same holds true under normal aerobic metabolism. The reversible oxidation of cysteine149 in GAPDH may be attributable to S-nitrosylation, S-thiolation (formed via an intermediate thyl radical), or the formation of sulfenic acid (37, 39–41). Various lines of evidence implicate protein disulfide isomerase, thioredoxin, glutaredoxin, and GSH in the reduction of the oxidized cysteine in GAPDH (20, 42, 43). Because the change in GSH levels after 1,25(OH)2D3 treatment is marginal (Table 1), and GSSG is not involved in GAPDH deactivation (44, 45), it is unlikely that the increase in GAPDH redox potential is secondary to the effect on glutathione redox potential. An inference compatible with our findings is that the 1,25(OH)2D3-induced increase in the redox potential of these two independent thiol redox couples is a manifestation of an overall shift in the cellular redox potential. In line with this mechanism is the comparable increase in the redox ratios of the two couples caused by treatment with the hormone, 41 ± 13% for GSSG:GSH as compared with 56 ± 5% for GAPDHox:GAPDHred. Treatment with 1,25(OH)2D3 also increased the cellular level of G6PD (Ref. 33, and Table 4). The level of this enzyme, which has a key role in the generation of NADPH via the pentose phosphate pathway, increases in response to various modes of oxidative challenge in mammalian cells (46). It is conceivable therefore, that its elevation in 1,25(OH)2D3-treated cells is an additional consequence of the prooxidant action of the hormone.

The prooxidant action of 1,25(OH)2D3 in MCF-7 cells could result from increased intracellular ROS production in the course of aerobic metabolism, and/or from changes in ROS handling. We show here (Table 3) that the ability to degrade H2O2 either by GPx or catalase or to handle organic peroxides by glutathione transferase (Table 4) is not impaired in 1,25(OH)2D3-treated cells. However, we have reported previously that treatment with 1,25(OH)2D3 inhibited the expression of one of the major constituents of the cellular defense system against ROS, the enzyme Cu/Zn SOD (9). This decrease could be one of the mechanisms underlying the prooxidant action of 1,25(OH)2D3. Indeed, it was previously shown that overexpression of SOD protects MCF-7 cells from injury caused by various ROS generating agents (47, 48). Decrease in SOD levels would cause a shift in the balance

DISCUSSION

The major finding of this study is that treatment of MCF-7 human breast cancer cells with 1,25(OH)2D3 brings about an increase in the redox potential of two unrelated thiol redox couples: GSSG-GSH and the oxidized and reduced forms of GAPDH. These changes in redox potential are not accompanied by changes in total glutathione or GAPDH levels. The increase in the GSSG:GSH ratio is not associated with modulations of the major GSH-consuming or -extrusion systems or failure of the GSH regenerating system. The latter is evidenced by the observation that 1,25(OH)2D3 does not affect the rate of degradation of exogenous H2O2 under conditions when GPx is rate-limiting. This in situ activity of the cultured cells depends on the combined and coordinated activities of GPx and glutathione reductase, which in turn depend upon the availability of GSH and NADPH, respectively.

Fig. 2. Effect of 1,25(OH)2D3 on the oxidized fraction of GAPDH in MCF-7 cells. Cells were treated with 1,25(OH)2D3 (100 nM) for 3 days. GAPDH activity in cell extracts was determined without or after treatment with DTT (40 mM, 1 h). Oxidized GAPDH is the increment in GAPDH activity after DTT treatment. Each bar represents one independent experiment. The data are presented as the mean of 2–4 replicate cultures. Right: bars, ± SE of the six experiments.

previous report that the hormone has no effect on GAPDH gene expression in MCF-7 cells (38). The effect of 1,25(OH)2D3 on the extent of thiol oxidation of the redox-sensitive enzyme, GAPDH, is compatible with the notion that 1,25(OH)2D3 increases the cellular redox potential in MCF-7 cells.
between superoxides and \( \text{H}_2\text{O}_2 \). Increased levels of superoxides can, in turn, cause increased oxidative damage attributable to interaction with NO to form the highly toxic peroxynitrite \( (49) \) and to increased availability of free iron that supports hydroxyl radical formation via the Fenton reaction \( (50) \).

Changes in the redox state could translate into reversible oxidation of cysteines in major proteins that determine cell fate, such as protein kinases, protein tyrosine phosphatases, and transcription factors \( (e.g., \text{Sp}1, \text{activator protein-1, nuclear factor xB, and p53; Refs.} 14–18, 51, 52) \). Key components of the apoptotic process, such as mitochondrial permeability transition pores and caspases, are also subject to redox regulation \( (53, 54) \). Oxidation of the cysteine in the active site of GAPDH may be considered a sensitive, easily accessible marker for these processes. It is noteworthy that the increase in the cellular redox potential reported here \( (3–8 \text{ mV}) \) is of the same order of magnitude as the redox change \( (15 \text{ mV}) \) that was shown to abolish the DNA-binding ability of the transcription factors activator protein-1 and nuclear factor xB \( (55) \). It was also shown that, at a GSSG:GSH ratio of 0.05 (similar to the values presented in this study), 50% of the cysteine residues in the DNA-binding domain of e-Jun were reversibly glutathionylated \( (56) \) and thus sensitive to small changes in the availability of free iron that supports hydroxyl radical formation via the Fenton reaction \( (50) \).

The potential reported here \( (3–8 \text{ mV}) \) is of the same order of magnitude as the oxidative potential \( (17.3–19.6 \text{ mV}) \) measured in a number of cell types. The potential difference observed between superoxides and \( \text{H}_2\text{O}_2 \). Increased levels of superoxides can, in turn, cause increased oxidative damage attributable to interaction with NO to form the highly toxic peroxynitrite \( (49) \) and to increased availability of free iron that supports hydroxyl radical formation via the Fenton reaction \( (50) \).

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