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Vitamin D Is a Prooxidant in Breast Cancer Cells[1]

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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$(OH)_2D_3$ augments the activity of anticancer agents that induce excessive reactive oxygen species generation in their target cells. This study aimed to find out whether 1,25$(OH)_2D_3$, acting as a single agent, is a prooxidant in cancer cells. The ratio between oxidized and reduced glutathione and the oxidation-dependent inactivation of glyceraldehyde-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$(OH)_2D_3$ (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$(OH)_2D_3$, as assessed by the rate of H_2O_2 degradation by MCF-7 cell cultures. Neither did treatment with 1,25$(OH)_2D_3$ 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$(OH)_2D_3$, which did not affect the total reduced enzyme activity. Treatment with 1,25$(OH)_2D_3$ 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$(OH)_2D_3$ 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, the hormone responsible for calcium and phosphate homeostasis. It is now recognized that 1,25$(OH)_2D_3$ also acts in a paracrine manner and is produced extrarenally 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$(OH)_2D_3$ has been commonly attributed to its direct cytostatic and cytotoxic effects on cancer cells (1). In addition, 1,25$(OH)_2D_3$ may exert some of its activity by cooperating with other anticancer agents. We and others have found that 1,25$(OH)_2D_3$ and its synthetic analogues increased the susceptibility of cancer cells to the cytotoxic/cytostatic action 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$(OH)_2D_3$ 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$(OH)_2D_3$ and these agents. Indeed, we found that the potentiation of the cytotoxic/cytostatic action of doxorubicin or the immune cytokines by 1,25$(OH)_2D_3$ 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$(OH)_2D_3$ (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 and do 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$(OH)_2D_3$ 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$(OH)_2D_3$ 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$(OH)_2D_3$ was obtained from Hoffmann-LaRoche Co.

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[3] The abbreviations used are: 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; ROS, reactive oxygen species; aminotriazole, 3-amino-1,2,4-triazole; BSO, buthionine sulfoximine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase.

(Nutley, NJ; a generous gift from Dr. M. Uskokovic). "Baker analyzed" H_2O_2 was from J. T. Baker (Philipsburg, NJ). Aminotriazole, mercaptosuccinic acid, BSO, and glutathione (reduced and oxidized forms) were purchased from Sigma Chemical Co. (St. Louis, MO). ICN Pharmaceuticals, Inc. (Costa Mesa, CA) supplied 2-vinylpyridine. All other reagents were of analytical grade.

Cell Culture. MCF-7 human breast cancer cells were cultured in Dulbecco's modified Eagle medium containing 4.5 g/liter glucose and supplemented with 10% FCS and antibiotics. Cells were subcultured twice weekly. Experiments were initiated by seeding 300,000 cells/60-mm Petri dish. Cultures were propagated for 5 days and treated with $1,25(OH)_2D_3$ or with the vehicle ethanol for the last 24–72 h of culture. The concentration of ethanol never exceeded 0.06%.

Determination of Glutathione. Cells were washed with ice cold PBS [phosphate buffer, 0.01 M; KCl, 2.7 mM; and NaCl 0.137 MM (pH 7.4)], scraped into cold PBS containing EDTA (5 mM) by gentle pipetting, and centrifuged for 20 s at 16,000 × *g* to obtain cell pellets, which were then resuspended in PBS containing EDTA (1 mM). Twenty mg/ml 5-sulfosalicylic acid was added to the cell suspensions, and after 15 min on ice, the protein precipitates were centrifuged for 1 min at 16,000 × *g*. Total and oxidized glutathione were determined in the supernatants by the glutathione reductase recycling assay as described by Griffith (24) and adapted to microtiter plates (25). GSSG was determined in the presence of 2-vinylpyridine to derivatize reduced glutathione (24). GSH was calculated as the difference between total and oxidized glutathione. Protein content in the sulfosalicylic acid precipitates was determined by the method of Lowry *et al.* (26).

Determination of Enzyme Activities. Cells were washed, scraped, and centrifuged as described above for glutathione determination. For GAPDH, cell pellets were resuspended in PBS containing sucrose (0.25 M) and immediately frozen [the addition of sucrose increases the stability of GAPDH in cell extracts (21)]. Before enzyme assay, the cells were thawed and pulse-sonicated (5 × 5 s; intensity 4) at 0°C by a Heat System Ultrasonics, Inc., sonicator (model no. W385). An aliquot of the sonicate was incubated with DTT (40 mM) for 1 h on ice. GAPDH activity was determined by following the reduction of NAD^+ with glyceraldehyde-3-phosphate as substrate in arsenate buffer (27). For glutathione reductase, cell suspensions in potassium phosphate buffer [12 mM (pH 7)] were sonicated, and enzyme activity was determined by following the oxidation of NADPH using GSSG as substrate (27). For glutathione *S*-transferase, cell suspensions in potassium phosphate buffer [12 mM (pH 7)] were sonicated, and enzyme activity was determined using 1-chloro-2,4-dinitrobenzene as substrate (28). For G6PD, cell suspensions in Tris-HCl buffer [15 mM (pH 8)] were sonicated, and enzyme activity was determined by following the reduction of $NADP^+$ to NADPH with glucose-6-phosphate as substrate (27).

H_2O_2 Degradation. Degradation of H_2O_2 by cells in culture was followed essentially as described by Makino *et al.* (29). In brief, the medium of cells cultured in 60-mm Petri dishes was replaced with 3 ml of H_2O_2 (0.2 mM) in PBS containing $CaCl_2$ (1 mM), $MgCl_2$ (0.5 mM), and glucose (4.5 g/liter). Cells were maintained at 37°C. Fifty-μl aliquots from the same Petri dish were collected at 5-min intervals up to 60 min, and H_2O_2 concentration was determined by the method of Thurman *et al.* (30).

RESULTS

The effect of $1,25(OH)_2D_3$ on glutathione homeostasis in MCF-7 human breast cancer cells is shown in Table 1. Treatment with $1,25(OH)_2D_3$ (10 or 100 nM) for 24 or 48 h brought about a highly significant increase in the ratio of GSSG:GSH ($P = 3.6 \times 10^{-5}$, by ANOVA of the results from four independent experiments presented in Table 1 in which 3–4 replicate cultures were treated with 100 nM $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$. For the sake of comparison, cultures of MCF-7 cells, prepared as described in Table 1, were exposed for 1 h to H_2O_2 (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 $1,25(OH)_2D_3$ was comparable with that after an acute exposure to a cytotoxic concentration of H_2O_2.

The increase in the GSSG:GSH ratio could be attributable to two conceptually different mechanisms. One possibility is that $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ on the activity of the enzymatic machinery responsible for glutathione homeostasis.

We first addressed this question by assessing the effect of $1,25(OH)_2D_3$ 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 H_2O_2. As described previously (29), the degradation of H_2O_2 followed first-order kinetics for at least 60 min. The relative contribution of catalase and GPx to the degradation of H_2O_2 was determined by the use of their specific inhibitors, aminotriazole (31) and

Table 1 *The effect of $1,25(OH)_2D_3$ on glutathione level and redox potential*

MCF-7 cells were plated and cultured as described in "Materials and Methods" and treated with $1,25(OH)_2D_3$ for the indicated times. The data are presented as the mean ± SD of 3–4 replicate cultures.

Experiment	Treatment	nmol/mg protein			GSSG/GSH[a] (×100)	ΔE (mV)[b]
		Total glutathione	GSH	GSSG[a]		
1	Control	36.0 ± 4.7	34.1 ± 4.5	0.97 ± 0.10	2.84 ± 0.09	
	1,25D (10^{-7} M; 48 h)	33.6 ± 4.1	31.3 ± 4.0	1.17 ± 0.03**	3.75 ± 0.39**	3.61
2	Control	25.3 ± 4.7	23.5 ± 4.2	0.90 ± 0.30	3.74 ± 0.49	
	1,25D (10^{-7} M; 48 h)	17.4 ± 4.6	15.9 ± 4.5	0.74 ± 0.05	4.67 ± 1.11	3.07
3	Control	25.5 ± 4.4	22.5 ± 3.9	1.46 ± 0.21	6.49 ± 0.46	
	1,25D (10^{-8} M; 48 h)	24.5 ± 1.8	21.2 ± 1.4	1.65 ± 0.21	7.75 ± 0.54*	2.26
	1,25D (10^{-7} M; 48 h)	24.6 ± 1.9	21.2 ± 1.9	1.72 ± 0.07	8.13 ± 0.79*	2.90
4	Control	28.5 ± 1.4	27.5 ± 1.3	0.52 ± 0.10	1.87 ± 0.29	
	1,25D (10^{-7} M; 24 h)	28.8 ± 3.5	27.5 ± 3.3	0.65 ± 0.10	2.35 ± 0.24*	2.93
	1,25D (10^{-7} M; 48 h)	27.3 ± 2.0	25.6 ± 1.9	0.87 ± 0.07***	3.39 ± 0.16***	7.68

[a] * $P < 0.05$; ** $P < 0.02$; *** $P < 0.005$ unpaired *t* test between 3–4 replicate cultures of $1,25(OH)_2D_3$-treated *versus* control cells.
[b] Difference between glutathione redox potential in $1,25(OH)_2D_3$-treated and control cultures, calculated according to Nernst's equation.

Table 2 *The contribution of catalase and GPx to H_2O_2 degradation by MCF-7 cells*

MCF-7 cells were plated and cultured for 5 days, and the degradation of H_2O_2 was monitored as described in "Materials and Methods." Aminotriazole (50 mM) and mercaptosuccinate (0.2 mM) were present 1 h before and during exposure to H_2O_2. The data are presented as the first order rate constants of H_2O_2 degradation.

Treatment[a]	$k \pm SE^b$ ($min^{-1} \times$ mg prot.$^{-1}$) $\times$ 100
Control	6.23 ± 0.30
No cells	0.55 ± 0.12
AT	1.96 ± 0.16
MS	4.30 ± 0.20
AT + MS	0.45 ± 0.09

[a] AT, aminotriazole; MS, mercaptosuccinate.
[b] Calculation of the first order rate constants was done by regression analysis of the exponential decay curves. The analysis of H_2O_2 degradation in the presence of aminotriazole was based on time points starting at 15 min coincubation with both the inhibitor and H_2O_2 to allow for complete irreversible inhibition of catalase (31).

Table 3 *The effect of 1,25(OH)$_2$D$_3$ on the degradation of H_2O_2 by MCF-7 cells*

MCF-7 cells were plated and cultured, treated with 1,25(OH)$_2$D$_3$ (100 nM) or vehicle for 72 h, and the degradation of H_2O_2 was monitored in the presence or the absence of aminotriazole (50 mM) as described in Table 2. The data are presented as the first order rate constants of H_2O_2 degradation.

Treatment[a]	$k \pm SE^b$ ($min^{-1} \times$ mg prot.$^{-1}$) $\times$ 100
Control	13.23 ± 0.43
1,25D (10 nM)	12.55 ± 0.42
1,25D (100 nM)	12.83 ± 0.39
AT	1.93 ± 0.11
AT + 1,25D (10 nM)	1.80 ± 0.19
AT + 1,25D (100 nM)	1.87 ± 0.17

[a] AT, aminotriazole.
[b] Calculation of the first order rate constants was done by regression analysis of the exponential decay curves. The analysis of H_2O_2 degradation in the presence of aminotriazole was based on time points starting at 15 min coincubation with both the inhibitor and H_2O_2 to allow for complete irreversible inhibition of catalase (31).

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 H_2O_2. Table 2 gives the first-order rate constants for H_2O_2 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 H_2O_2 in the absence of cells. It may thus be inferred that the combined activity of catalase and GPx fully accounts for the degradation of H_2O_2 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)$_2$D$_3$ (100 nM) had no effect on H_2O_2 degradation in the presence or absence 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 H_2O_2 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 H_2O_2, 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)$_2$D$_3$ 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)$_2$D$_3$ (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)$_2$D$_3$-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)$_2$D$_3$ on glutathione transferase activity in MCF-7 cell extracts.

Modulation of glutathione consumption and efflux could also affect the glutathione redox state. Fig. 1*A* 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

Table 4 *Effect of 1,25(OH)$_2$D$_3$ on glutathione reductase, glutathione S-transferase, and G6PD*

MCF-7 cells were plated and cultured as described in Table 1 and treated with 1,25(OH)$_2$D$_3$ (100 nM) for the last 48 h of culture. Cells were harvested, cell extracts were prepared, and enzyme activities were assayed as described in "Materials and Methods."

	nmol/min/mg protein (mean ± SE)[a]	
	Control	1,25(OH)$_2$D$_3$
Glutathione reductase	217 ± 12	209 ± 4
Glutathione *S*-transferase	43.0 ± 2.8	43.4 ± 5.5
G6PD	346 ± 30	478 ± 48[b]

[a] The number of experiments were 3, 4, and 5 for glutathione reductase, glutathione *S*-transferase, and G6PD, respectively.
[b] $P = 0.004$; paired *t* test, 1,25(OH)$_2$D$_3$-treated *versus* control cells.

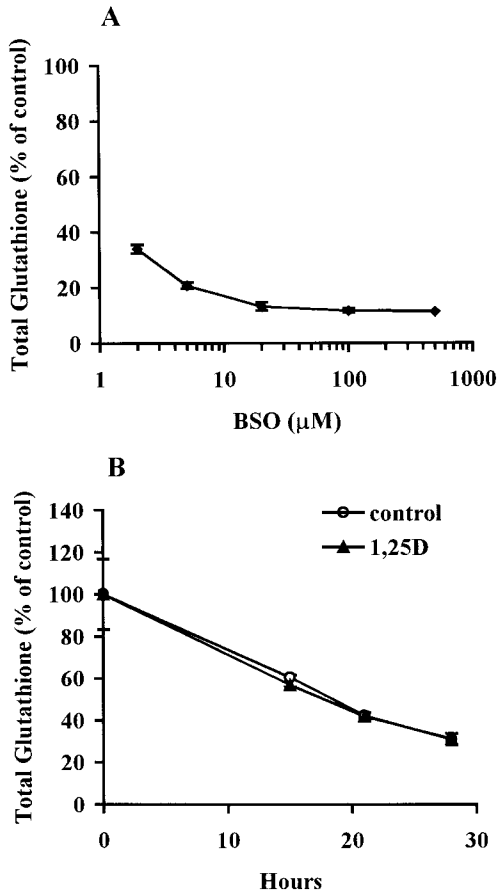

Fig. 1. Effect of 1,25(OH)$_2$D$_3$ on cellular glutathione levels in the presence of BSO. *A*, MCF-7 cell cultures were treated with BSO at various concentrations for 48 h. *B*, cells were treated with 1,25(OH)$_2$D$_3$ (100 nM) for 48 h and then with BSO (20 μM) for the indicated times. The data are presented as the mean ± SD of triplicate cultures (in some cases, the *error bars* are smaller than the *symbols* and cannot be seen).

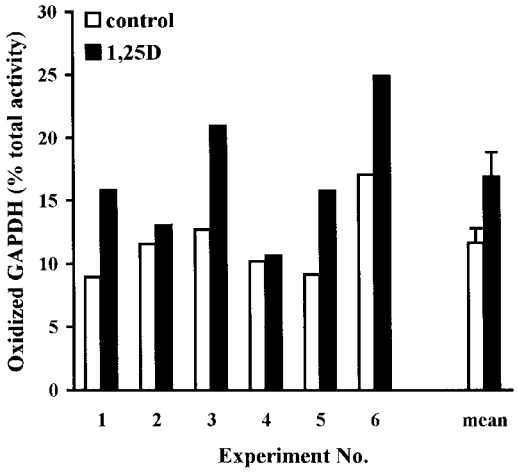

Fig. 2. Effect of $1,25(OH)_2D_3$ on the oxidized fraction of GAPDH in MCF-7 cells. Cells were treated with $1,25(OH)_2D_3$ (100 nM) for 3 days. GAPDH activity in cell extracts was determined without or after treatment with DTT (40 mM, 1 h). *Oxidized GAPDH* is the increment in GAPDH activity after DTT 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.

dose-dependent and saturates at a concentration of 20 μM (Fig. 1*A*). $1,25(OH)_2D_3$-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. 1*B*). It is evident that the rate of decline in glutathione is not affected by $1,25(OH)_2D_3$. 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 $1,25(OH)_2D_3$ 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 further recovery of GAPDH activity at higher DTT concentrations or after longer incubation periods). We assume that GAPDH activity after treatment with DTT represents the total level of cellular GAPDH. We found that reduction with DTT increased GAPDH activity in MCF-7 cell extracts. This increase (13.2 ± 1.5%, mean ± SE; $P = 4.7 \times 10^{-4}$; paired *t* test of six independent experiments) represents the *in situ* reversibly oxidized GAPDH. Using this experimental set up, we examined the effect of $1,25(OH)_2D_3$ on total GAPDH levels and the ratio between the oxidized and reduced fraction of the enzyme (GAPDHox;GAPDHred). The data depicted in Fig. 2 clearly show that treatment with the hormone (72 h; 100 nM), significantly increased the GAPDHox;GAPDHred ratio in MCF-7 cells. The average increase in the GAPDH redox ratio was 56 ± 5.3% (mean ± SE; $P = 0.014$; paired *t* test). In the same experiments, there was no difference in total GAPDH activity (assayed after reduction with DTT) as a result of treatment with $1,25(OH)_2D_3$ (1.27 ± 0.13, mean ± SE; in control *versus* 1.33 ± 0.15 μmol/min × mg protein in $1,25(OH)_2D_3$-treated cultures). This finding is in agreement with a previous report that the hormone has no effect on *GAPDH* gene expression in MCF-7 cells (38). The effect of $1,25(OH)_2D_3$ on the extent of thiol oxidation of the redox-sensitive enzyme, GAPDH, is compatible with the notion that $1,25(OH)_2D_3$ increases the cellular redox potential in MCF-7 cells.

DISCUSSION

The major finding of this study is that treatment of MCF-7 human breast cancer cells with $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ does not affect the rate of degradation of exogenous H_2O_2 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. A simple stoichiometric calculation shows that the amount of GSH consumed during the degradation of H_2O_2 in these experiments (Tables 2 and 3) equals ~14-fold the total glutathione pool (Table 1). Because $1,25(OH)_2D_3$ 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 cys^{149} in GAPDH may be attributable to *S*-nitrosylation, *S*-thiolation (formed via an intermediate thyil 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$-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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$-treated cells is an additional consequence of the prooxidant action of the hormone.

The prooxidant action of $1,25(OH)_2D_3$ 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 H_2O_2 either by GPx or catalase or to handle organic peroxides by glutathione transferase (Table 4) is not impaired in $1,25(OH)_2D_3$-treated cells. However, we have reported previously that treatment with $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$. 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

between superoxides and H_2O_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.*, Sp1, activator protein-1, nuclear factor κB, 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 mV) is of the same order of magnitude as the redox change (15 mV) that was shown to abolish the DNA-binding ability of the transcription factors activator protein-1 and nuclear factor κB (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 c-Jun were reversibly glutathionylated (56) and thus sensitive to small changes in the cellular redox potential. These and similar findings indicate that perturbations in the cellular redox state similar to those found in the present study can indeed affect the function of redox-sensitive proteins. Our finding that the effect of $1,25(OH)_2D_3$ on the cellular redox state is similar to that caused by an hour of exposure to the potent oxidant H_2O_2, provides additional evidence of the biological significance of the action of this hormone.

The anticancer activity of $1,25(OH)_2D_3$ is associated with induction of differentiation, cell cycle arrest, and apoptosis, and with sensitization of cancer cells to the cytotoxic action of some host anticancer agents and therapeutic modalities. The data of this study lead us to present the notion that an increase in the cellular redox potential plays a role in the various anticancer actions of active vitamin D metabolites and analogues.

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