Carnosic Acid Potentiates the Antioxidant and Prodifferentiation Effects of 1α,25-Dihydroxyvitamin D₃ in Leukemia Cells but Does Not Promote Elevation of Basal Levels of Intracellular Calcium

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ABSTRACT

Differentiation therapy of cancer remains an only partially attained goal. Agents currently under active investigation include derivatives of vitamin D, modeled on its physiological hormone form, 1α,25-dihydroxyvitamin D₃ (1,25D₃), but the calcemic effects of these compounds preclude their use in the clinic. An approach that may obviate this problem is to combine 1,25D₃, or its derivatives with other agents that increase the antineoplastic effects of low, nontoxic concentrations of vitamin D compounds. We have recently used the plant-derived polyphenolic antioxidant, carnosic acid (CA), to demonstrate an increase in the differentiating action of 1,25D₃, on human leukemia cells under these conditions (M. Danilenko et al., JNCI, 93: 1224–1233, 2001). We now show that treatment of HL60-G cells with either CA or 1,25D₃, alone resulted in a decrease in the intracellular levels of reactive oxygen species. Furthermore, the combination of 10 μM CA and a low concentration of 1,25D₃ (1 nM) produced an enhanced antioxidant effect, which correlated with the potentiation of monocytic differentiation. Other plant antioxidants tested (curcumin, silibinin, and the organoselenium antioxidant ebselen) also potentiated differentiation induced by 1,25D₃, although alone, they had only minor differentiating effects. Differentiation induced by CA/1,25D₃, combinations was associated with increased intracellular glutathione content, whereas buthionine sulfoxime decreased both differentiation and the cellular glutathione content. This combination also enhanced the activation of the Raf-mitogen-activated protein/extracellular signal-regulated kinase kinase-extracellular signal-regulated kinase mitogen-activated protein kinase module and increased the binding of the activator protein-1 (AP-1) transcription factor to its cognate DNA element in the promotor regions of vitamin D receptor gene, suggesting that the mechanism of potentiation is at least in part attributable to induction and activation of components of this mitogen-activated protein kinase pathway. Cell treatment with a high concentration of 1,25D₃, (100 nM) resulted in a substantial elevation of basal intracellular calcium concentration. In contrast, importantly for an eventual clinical application of these studies, the potentiation action of CA on differentiation induced by a low concentration of 1,25D₃, (1 nM) was not accompanied by an elevation of basal intracellular calcium concentration. These findings suggest that combinations of CA with derivatives of vitamin D should be evaluated for use in differentiation therapy of myeloid leukemias.

INTRODUCTION

The goal of differentiation therapy of cancer is to arrest the growth of malignant cells by inducing normalization of cellular phenotypes without damage to normal tissues. A notable example of the success of this approach is provided by the use of vitamin A derivatives, which are in varying stages of development for treatment of malignant diseases, e.g., ATRA ³ is particularly effective in the treatment of acute promyelocytic leukemia (reviewed in Refs. 1 and 2). Similarly, 1,25D₃ and its derivatives are currently under investigation as differentiating agents in a variety of tumor types and seem especially suited for clinical applications: (a) 1,25D₃ is a hormone with a known physiological function in the control of calcium homeostasis; (b) there is substantial epidemiological evidence that levels of circulating 1,25D₃ near the top of the physiological range play a part in the reduction of incidence of the common human cancers that affect the female breast, prostate, and colon (3); and (c) differentiation and apoptosis-inducing effects of 1,25D₃, have been demonstrated in neoplastic cells established in culture from these and other tissues (reviewed in Ref. 4), showing that under appropriate conditions, 1,25D₃ can indeed control the growth of these cells.

The well-known limitation to the therapeutic use of 1,25D₃, is its hypercalcemic effect. When the hypercalcemia is sufficiently prolonged and severe, widespread calcifications take place in tissues. Current attempts to overcome this problem focus on the synthesis of analogues of 1,25D₃, which retain the prodifferentiation activities but have lower calcemic effects (e.g., Ref. 5). However, although various Phase I/II trials have been conducted (e.g., Refs. 6–10), these vitamin D analogues have as yet not been successfully used for the treatment of cancer, including myeloid leukemias. An alternative approach is to combine nonhypercalcemic concentrations of 1,25D₃, or its analogues with compounds that have different mechanisms of action, e.g., increased antitumor activity has been reported when dexamethasone or cytotoxic agents, such as paclitaxel, were combined with 1,25D₃ (11–13), whereas several plant-derived antioxidant compounds, such as polyphenols curcumin (14, 15) and silibinin (16), and carotenoids lycopene and ß-carotene (17) were found to potentiate the differentiating and antiproliferative actions of 1,25D₃, on leukemic cell lines. We have recently reported a marked potentiation of the 1,25D₃ induced differentiation of HL60 human myeloid leukemia cells by CA, a polyphenol derived from the plant rosemary (Rosmarinus officinalis; Refs. 18 and 19). We also found that this potentiation relates to the ability of CA to enhance a program of gene expression consistent with monocytic differentiation (19). In the present study, we investigated the mechanism of the synergy between 1,25D₃ and CA and found that 1,25D₃ and CA cooperatively decreased the levels of ROS in this system, increased total cellular glutathione content, activated the Raf/MEK/ERK MAPK module, and enhanced the binding of AP-1 to its DNA response element. Furthermore, we demonstrated that, in contrast to induction of differentiation with a high concentration of 1,25D₃ (100 nM), which had a differentiating effect accompanying a considerable elevation of basal intracellular calcium

³The abbreviations used are: ATRA, all-trans retinoic acid; 1,25D₃, 1α,25-dihydroxyvitamin D₃; TPA, 12-O-tetradeconoxyphorbol 13-acetate; CA, carnosic acid; ROS, reactive oxygen species; [Ca²⁺], intracellular calcium concentration; DCFH-DA, 5,6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; [MLP, N-formyl-methionyl leucyl-phenyalanine; AP-1, activator protein; bVDNR, human vitamin D receptor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; GSH, reduced glutathione; BSO, buthionine sulfoxime; MAPK, mitogen-activated protein kinase; KCP, tripeptide lysine-cysteine-arginine.

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levels in HL60 cells, the combination of a low (1 nm) 1,25D3 concentration with CA only moderately affected the basal [Ca2+]i.

**MATERIALS AND METHODS**

**Chemicals and Antibodies.** CA was obtained from Alexis Biochemicals (Laulenfingen, Switzerland). Curcumin, ebolen, silibinin, 1-buthionine, BSO, FMLP, DCFH-DA, TPA, GSH, glutathione reductase from bakers yeast, 5,5′-dithiobis(2-nitrobenzoic acid), 5-sulfosalicylic acid, and NADPH were purchased from Sigma (St. Louis, MO). Complete protease inhibitor cocktail was from Roche Molecular Biochemicals (Mannheim, Germany). Poly d[d(C)] was from Roche Diagnostics (Mannheim, Germany). NP40 was from Calbiochem-Novabiochem Corp. (San Diego, CA). 1,25D3 was a gift from Dr. Milan Uskokovic (BioXell, Nutey, NY). [γ-32P]ATP was purchased from NEN Life Science Products, Inc. (Boston, MA). The antibodies against Raf-1 (c-12, rabbit polyclonal), MEK-1 (c-18, rabbit polyclonal), ERK1/2 (K-23, rabbit polyclonal), and p90RSK (c-21, rabbit polyclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies used to detect phospho-Raf (Ser 259), phospho-MEK (Ser 217/221), phospho-ERK (Thr 202/Tyr 204), and phospho-p90RSK (Ser 381), all rabbit polyclonal antibodies, were purchased from Cell Signaling Technology (Beverly, MA). Anticalreticulin antibody was purchased from Affinity Bioreagents (Golden, CO). Horseradish peroxidase-conjugated antirabbit IgG and antimouse IgG were obtained from Santa Cruz Biotechnology. Anti-CD14 (MY4-RD-1) and anti-CD11b (MO1-FITC) antibodies were obtained from Coulter Corp. (Brea, CA). Stock solutions of CA, curcumin, silibinin, ebolen (10 mM each), and 1,25D3 (0.25 mM) were prepared in absolute ethanol.

**Cell Culture and Proliferation Assay.** HL60-G cells (20), a subclone of human promyelocytic leukemia HL60 cells (21), were routinely cultured at 37°C in RPMI 1640 (Mediatech, Washington, D.C., or Biological Industries, Beit Haemek, Israel), supplemented with 10% heat-inactivated, iron-enriched bovine calf serum (HyClone, Logan, UT). Cell culture waspassaged two to three times weekly to maintain a log phase growth. Cells were seeded into fresh culture medium at 0.5–1×105 cells/ml in 25 cm2 tissue culture flasks and incubated with test agents for 24–48 h. To demonstrate the enhancement of differentiation and growth inhibition induced by 1,25D3, cells were treated with a low concentration of this inducer (1 nm) in the presence of other agents, whereas 100 nm 1,25D3 was used to illustrate the maximal effect. Cell growth was estimated by counting cells with a Coulter Counter after dilution in Isoton-II (Coulter Electronics, Hialeah, FL). Cell viability was determined using trypsin blue (0.25%) exclusion. To determine the maximal subtoxic concentration of each antioxidant, the cells were cultured for 48 h in the presence of graded concentrations of the compound, and cell viability was measured as described above.

**Determination of Markers of Differentiation.** Aliquots of 1×106 cells were harvested, washed twice with PBS, and suspended in 10 μl of PBS. The cell suspensions were incubated for 45 min at room temperature with 0.5 μl of MY4-RD-1 and 0.5 μl of MO1-FITC (1:20 dilution of the stock antibodies) to analyze the expression of surface cell markers CD14 and CD11b, respectively (19). The cells were then washed three times with ice-cold PBS and resuspended in 1 ml of PBS. Two-parameter analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Iso-typic mouse IgG was used to set threshold parameters.

**Cell Experiments.** All procedures were carried out at 4°C. Cells (1–2×106) were harvested and washed twice with ice-cold PBS. Whole-cell extracts were prepared essentially as described previously (19). Washed cell pellets were solubilized with a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium PPi, 1 mM β-glycerophosphate, 1 mM Na2VO3, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μg/ml aprotinin. Equal amounts of 3× SDS sample buffer containing 150 mM Tris-HCl (pH 6.8), 30% glycerol, 3% SDS, 1.5 mg/ml bromphenol blue dye, and 100 mM DTT were then added to each sample. Nuclear extracts were prepared by the procedure described before (19, 22) with minor modifications. Briefly, cell pellets were resuspended in 0.5 ml of ice-cold hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and Complete protease inhibitor cocktail]. The cells were kept on ice for 10 min to allow them to swell, vortexed for 10 s, and centrifuged at 16,000×g for 30 s. Supernatant was discarded, and the pellet was resuspended in 50 μl of nuclear extraction buffer [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and Complete protease inhibitor cocktail], placed on ice for 20 min, and centrifuged at 16,000×g for 15 min. The supernatant was saved as the nuclear extract and stored at −80°C.

**Western Blotting.** Equal amounts of whole-cell extracts (40 μg of protein) were separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were blocked with 5% milk in Tris-buffered saline/0.1% Tween 20 for 1 h, subsequently blotted with primary antibodies, and then the membranes were blotted with a horseradish-linked secondary antibody for 1 h. The protein bands were visualized with a chemiluminescence assay system (Amersham). The protein loading of the gel and efficiency of the transfer were controlled by stripping the membrane and reprobing for calreticulin, a constitutively expressed protein in HL60 cells. The absorbance of each band was quantitated using an image quantitator (Molecular Dynamics, Sunnyvale, CA).

**Electrophoretic Mobility Shift Assay.** AP-1 binding to its cognate DNA element (TPA-response element) in a 1,25D3-responsive gene, hVDR, was evaluated as described previously (23) with the following modifications. Double-stranded oligonucleotides from promoter regions of hVDR containing the proximal (−77 to −97 relative to the transcription start site) binding site for AP-1 (5′-CTGCCAAGAGACTGGGAC-3′) and the distal (−1023 to −1043) hVDR-AP-1#2 (5′-GATTAGTGGATCATGGTGG-3′) were synthesized by the Molecular Resource Facility of the New Jersey Medical School. The reference sequence accession number for hVDR in the GenBank for National Center for Biotechnology is AB002157. Nuclear extracts (10 μg of protein) were preincubated with 0.02 A260 units of poly (d[d(C)] for 15 min on ice in buffer containing 50 mM Tris-HCl (pH 7.9), 10 mM MgCl2, 1 mM EDTA, 100 mM KCl, 2 mM DTT, 20% (volume/volume) glycerol, and 0.1% (w/v) protease inhibitor cocktail NP40. The extracts were then incubated for an additional 30 min at 30°C. The reaction was stopped by resuspending two to four nuclei in 50–60 pg (50,000–75,000 cpm) of 32P-labeled, double-stranded oligonucleotide. Specificity of the AP-1 binding was estimated by competition with a 10× molar excess of the unlabeled double-stranded “self” nucleotide (see above) or unrelated double-stranded nucleotide containing the Sp1 response element (5′-ATTCCATGGCGGCGGCGGCGG-3′) added to parallel samples during the preincubation period. To examine the effects of oxidation of nuclear proteins on their AP-1 DNA-binding activity, samples of nuclear extracts were preincubated with the oxidizing agent diamide (20 mM). The complexes were separated on 4% polyacrylamide gel under non-denaturating conditions with a constant current of 20–25 mA for 3–4 h at 4°C. The gel was dried and exposed overnight to Kodak X-Omat LS film.

**Measurement of Intracellular Peroxides by Flow Cytometry.** The intracellular peroxide levels were determined using the oxidation-sensitive fluorescent probe DCFH-DA (24, 25). Intracellular peroxides oxidize this probe to a highly fluorescent compound, DCF. Cells (1×106/ml) were harvested at the time points indicated and washed with HEPES-buffered HBSS. Cells were then loaded with 5 μM DCFH-DA for 15 min with horizontal agitation in a shaking water bath at 37°C. In some experiments, after loading with DCFH-DA, cells were washed with PBS and incubated for an additional 30 min with 0.01–1 mM H2O2 under the same conditions. The fluorescence intensity was measured with a FACSCalibur flow cytometer (Becton Dickinson). For each analysis, 10,000 events were recorded.

**Superoxide Anion Measurement.** The production of superoxide (O2−) was measured in a 96-multifwell format by the superoxide dismutase-inhibitable reduction of cytochrome c as described previously (18). Cells were washed in HBSS and suspended (2.5 ×103 cells/well) in 100 μl of HBSS buffered with 150 mM NaCl, 1 mM Na2EDTA, 1 mM Na2EGTA, 1% Triton X-100, 2.5 mM sodium PPi, 1 mM β-glycerophosphate, and 100 mM DTT. The reduction of cytochrome c was monitored by the addition of either TPA (100 mM) or FMLP (10 μM), and the reduction of cytochrome c was monitored at 550 nm (650-nm reference wavelength) for 30–40 min at 37°C in a VERSAmax microplate spectrophotometer ( Molecular Devices, Menlo Park, CA). The maximal rates of superoxide generation were determined and expressed as nmol O2−/106 cells/min using extinction coefficient E550 = 21 μM−1cm−1.

**Assay for Glutathione.** Cells (2×106) were collected by centrifugation (1,000×g for 5 min), washed with ice-cold PBS, and resuspended in 200 μl of 5% 5-sulfosalicylic acid. After 15 min on ice with intermittent vortexing, the suspension was centrifuged at 16,000×g for 5 min to remove protein precipitates. Total glutathione was determined in the supernatants by the
glutathione reductase recycling assay as described by Griffith (26) with minor modifications (27).

**Determination of [Ca^{2+}]**. Calcium assay was performed as described previously (28) with minor modifications. Briefly, after a 96-h incubation, cells were harvested, washed with PBS, and resuspended in HBSS containing 10 mM HEPES and 1 mg/ml BSA. Cells were incubated with 2 μM fura-2 a.m. (Molecular Probes, Inc., Eugene, OR) for 30 min at 25°C. Before [Ca^{2+}] measurements, 0.5–1 × 10^6 cells were aliquoted to microfuge tubes, centrifuged for 5 s, resuspended in 100 μl of HEPES and 1 mg/ml BSA, and injected into cuvettes containing 1.9 ml of the same solution. Data were collected at 2-s intervals at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Fluorescence was monitored at 37°C with constant stirring in a Perkin-Elmer Model 50-LS fluorescence spectrophotometer. Basal [Ca^{2+}] and the peak Ca^{2+} response of cells stimulated with 10 μM fMLP were measured. Calibration of the signal was achieved by exposing cells to 0.1% Triton X-100 in the presence of saturating Ca^{2+} or HBSS containing 15 mM EGTA (pH 8.0). Autofluorescence of solutions, drugs, and fura-2-free-treated cells was subtracted from the fluorescence spectra before [Ca^{2+}] calculations (28, 29).

**Statistical Analysis.** All experiments were repeated at least three times. The significance of the differences between the means of the various subgroups was assessed by two-tailed Student’s t test. The computations were performed with an IBM-compatible personal computer using Microsoft EXCEL and GraphPad Prism 3.0 (GraphPad Software, San Diego, CA) programs.

**RESULTS**

**CA and 1,25D3 Cooperate As Antioxidants in HL60-G Cells.** CA and related polyphenols have been known to act as both antioxidant and pro-oxidant in different biological systems (30–32). Therefore, we set out to investigate whether the potentiating effect of this polyphenol on cell differentiation (18, 19) is accompanied by any detectable changes in the levels of ROS in HL60-G cells. For these experiments, the cells were incubated with 0, 5, and 10 μM CA alone for 96 h, followed by measurements of the levels of intracellular peroxides by flow cytometry using the fluorescence oxidation-sensitive probe DCFH. As shown in Fig. 1A, CA produced a concentration-dependent decrease in the intracellular levels of ROS, as compared with untreated control cells. Interestingly, 1,25D3 alone induced an even greater decrease in ROS levels (Fig. 1B; see also Table 1), and cell treatment with a combination of 10 μM CA with 1 nM 1,25D3 resulted in a cooperative effect in at least a large part of the cell population (Fig. 1C). The data summarized in Table 1 indicate that although the combined antioxidant effect of CA + 1,25D3 is bimodal (Fig. 1C), its overall magnitude is similar to that obtained with 100 nm 1,25D3 alone. Concurrently, a similar extent of cell differentiation was observed under these treatments (Fig. 7, C and D). The capacity of both CA and 1,25D3 to reduce ROS levels increased with time reaching a saturation by 96 h (data not shown).

To further evaluate the antioxidant effects of CA and 1,25D3, after DCFH loading, control cells and cells treated with 10 μM CA, 1 nM 1,25D3, and the combination of 1 nM 1,25D3 and 10 μM CA or 100 nm 1,25D3 were challenged with increasing concentrations of H2O2 (0–1 mM). The results demonstrate that in the CA-treated cells, a 30-min incubation with H2O2 induced less pronounced increases in ROS levels, as compared with control cells (Fig. 2). Treatment with 1 nM 1,25D3 had a minor effect on the H2O2-induced elevation of ROS, whereas a high concentration of 1,25D3 (100 nm) substantially inhibited the oxidant action. Importantly, a similar inhibition of ROS production was obtained in cells incubated with 1 μM 1,25D3 together with CA. Taken together, these results indicate that the two agents are capable of protecting the cells against oxidative stress and that there is correlation between the differentiating effects of 1 nM 1,25D3 + CA and 100 nm 1,25D3, and the antioxidant effects of these corresponding treatments.

**Diverse Compounds with Antioxidant Action also Potentiate Differentiation Induced by 1,25D3.** If the antioxidant properties of CA have a role in potentiation of differentiation by 1,25D3, other antioxidants would be expected to increase the prodifferentiation effects of 1,25D3 to some extent. We therefore tested several such compounds at their maximal subtoxic concentrations (viability > 90%) in HL60-G cells and found that although the synthetic antioxidants ebselen (Fig. 3), hydroxyurea, and diphenylene iodonium (data not shown) had slight to moderate potentiating effects on 1,25D3-induced differentiation, these effects were more marked when plant polyphenols curcumin and silibinin were used (Fig. 3). Conversely, the pro-oxidant BSO, which depletes cellular glutathione by inhibiting γ-glutamylcysteine synthetase (33), moderately decreased the differentiating effect of 1,25D3 alone and markedly inhibited the enhanced differentiation induced by its combination with CA (Fig. 4A). On the other hand, cell treatment with CA resulted in a substantial increase in the intracellular glutathione levels, which was further elevated by adding 1 μM 1,25D3, and both these increases were inhibited by BSO (Fig. 4B). These findings indicate that reducing

<table>
<thead>
<tr>
<th>1,25D3</th>
<th>CA</th>
<th>High ROS-containing cells (%)</th>
<th>Mean fluorescence intensity</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100.0 ± 3.2</td>
<td>104.4 ± 10.8</td>
</tr>
<tr>
<td>0</td>
<td>10 μM</td>
<td>89.3 ± 10.4</td>
<td>71.9 ± 8.1</td>
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<tr>
<td>1 nM</td>
<td>0</td>
<td>80.9 ± 7.2 a</td>
<td>62.7 ± 3.5 b</td>
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<tr>
<td>1 nM</td>
<td>10 μM</td>
<td>52.4 ± 8.6 a</td>
<td>34.3 ± 8.0 b</td>
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<tr>
<td>100 nm</td>
<td>0</td>
<td>46.2 ± 4.7 c</td>
<td>37.8 ± 3.8 b</td>
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a P < 0.05
b P < 0.01 between samples of treated cells versus control cells.
The Raf/MEK/ERK MAPK Module Is Activated by CA in 1,25D₃-induced Differentiation. It has been shown in several laboratories that ERK 1/2 MAPK pathway participates in 1,25D₃-induced differentiation of HL60 cells (34–36). To initiate studies of the mechanisms of the potentiating effect of CA on this form of differentiation, we examined the activation of several components of this pathway, as evidenced by the level of phosphorylation of these signaling proteins. Consistent with the suggested role of this pathway in differentiation, in cells treated with CA alone, which showed borderline differentiation, a minimal increase was noted in phosphorylation of Raf-1 and MEK-1 (Fig. 5). Interestingly, phosphorylation appeared to stabilize these proteins, because the total protein levels also increased after exposure to CA.

More pronounced increases in activation of each member of the ERK 1/2 cascade, as well as of RSK-1, one of the known downstream targets of this pathway, were noted when CA was combined with 1,25D₃, and these increases were of similar magnitude as those that resulted from exposure to a high (100 nM) concentration of 1,25D₃, whereas calreticulin, a constitutively expressed cellular component, showed no changes in its level of expression (Fig. 5). Thus, the data suggest that enhanced activation of the ERK 1/2 pathway is associated with the potentiation by CA of monocytic differentiation of HL60 cells.

A Possible Role of the AP-1 Transcription Factor in the Enhancement of Differentiation by CA. Activation of the ERK cascade has been associated with increased functional activity of AP-1 transcription factor (reviewed in Refs. 37 and 38), and recent studies have indicated the role of AP-1 activation in myeloid differentiation of leukemic cells (23, 39–41), and in the expression of the VDR gene (42). Importantly, AP-1 is regulated by cellular redox status (43). Therefore, we determined whether the enhancement of 1,25D₃-induced monocytic differentiation of HL60 cells was accompanied by changes in the DNA-binding capacity of nuclear proteins to the AP-1 motifs present in the promoter of the human VDR gene (GenBank accession no. AB002157). Consistent with the data reported previously (23), cell treatment with 1,25D₃ resulted in increases in binding to both these AP-1 motifs. This effect was pronounced in cells treated with 100 nM 1,25D₃, whereas at 1 nM, this inducer showed only a moderate effect (Fig. 6). However, combining 1 nM 1,25D₃ with CA, which was also relatively ineffective in this assay, caused a substantial elevation of AP-1 binding activity. Elimination of binding because of competition with unlabeled AP-1 response element probe but not with a mutated AP-1 sequence or a “nonsite” probe (Sp-1 response element) indicates the specificity of the AP-1 gel-shift assay in this system. These data suggest that the AP-1 transcription factor regulates genes that participate in the CA-enhanced program of monocytic differentiation.

CA Does Not Augment the 1,25D₃-induced Rise of Basal Cytosolic Calcium Levels. Several studies have shown that the 1,25D₃-induced monocytic differentiation of myeloid leukemia cells is ac-

![Fig. 2. CA and 1,25D₃ reduce intracellular ROS levels in H₂O₂-treated HL60-G cells. Control cells (1 × 10⁶ cells; A) and those treated with 10 μM CA (B) or 100 nM 1,25D₃ (C) were incubated with 5 μM DCFH-DA for 15 min at 37°C and washed once with HEPES-buffered HBSS. Cells were treated for an additional 30 min with the indicated concentrations of H₂O₂. Fluorescence intensity of the oxidized product (DCF) was determined with a flow cytometer. A representative of three similar experiments is shown. In D, quantitation is presented (mean fluorescence intensity ± SD, n = 4) of the experiments illustrated in A–C, as well as of experiments in which 1 nM 1,25D₃, alone or together with 10 μM CA, was used in analogous assays.

![Fig. 3. Diverse antioxidants potentiate cell differentiation induced by 1,25D₃. Cells were incubated with the indicated compounds for 48 h followed by analysis of the expression of monocytic differentiation markers CD11b and CD14 by flow cytometry, as described in “Materials and Methods.” The means ± SE of at least four experiments are shown. The experimental groups marked with an asterisk were significantly increased compared with cells treated with 1,25D₃ alone (*P < 0.05).

![Fig. 4. The effects of glutathione-depleting agent, buthionine sulfoximine, on differentiation and glutathione levels in cells treated with 1,25D₃, and its combination with CA. HL60-G cells were incubated with the indicated compounds for 96 h followed by analysis of CD11b and CD14 expression by flow cytometry and the measurement of glutathione by an enzymatic assay, as described in “Materials and Methods.” In A, buthionine sulfoximine inhibits differentiation. Determination of CD14 and CD11b double positive cells was carried out by flow cytometry. The means ± SE of three experiments are shown. In B, buthionine sulfoximine blocks increases in intracellular glutathione levels. Glutathione was determined by an enzymatic assay, as described in “Materials and Methods.” The means ± SE of four experiments are shown.
compounded both by an increase in basal cytosolic calcium levels and the expression of fMLP receptors, which mediate the chemotactic peptide-induced transient \([\text{Ca}^{2+}]_i\) elevations (28, 29, 44–47). Because disturbances of calcium homeostasis may have profound implications for any potential use of CA/1,25D3 in differentiation therapy of myeloid leukemia, we determined whether along with its enhancement of the differentiating and antiproliferating effects of 1,25D3, CA can also potentiate the ability of this inducer to affect cytosolic calcium levels. Changes in both basal \([\text{Ca}^{2+}]_i\) and its elevations in response to fMLP were monitored in HL60-G cells treated for 96 h with 1,25D3, CA, and their combination using the fluorescent calcium probe fura-2. Cells treated with 100 nM 1,25D3 showed a substantial increase \((P < 0.01)\) in the basal \([\text{Ca}^{2+}]_i\) as compared with untreated control cells (Fig. 7), whereas neither 1 nM 1,25D3 nor 10 \(\mu\)M CA had a significant effect. The addition of 10 \(\mu\)M fMLP to control or to CA-treated cells did not affect their basal \([\text{Ca}^{2+}]_i\), whereas in cells incubated with 1,25D3, this peptide induced a transient elevation of cytosolic calcium. These data are consistent with the induction of chemotactic peptide receptors in 1,25D3-differentiated HL60 cells (18, 48, 49). The peak values of the fMLP-stimulated \([\text{Ca}^{2+}]_i\) rises depended on 1,25D3 concentration (Fig. 7). The addition of 10 \(\mu\)M fMLP to control or to CA-treated cells did not affect their basal \([\text{Ca}^{2+}]_i\), whereas in cells incubated with 1,25D3, this peptide induced a transient elevation of cytosolic calcium. These data are consistent with the induction of chemotactic peptide receptors in 1,25D3-differentiated HL60 cells (18, 48, 49). The peak values of the fMLP-stimulated \([\text{Ca}^{2+}]_i\) rises depended on 1,25D3 concentration (Fig. 7). The addition of CA to 1 nM 1,25D3 during 96-h incubations resulted in the enhanced calcium response to fMLP, which was comparable with that observed in the 100 nM 1,25D3-exposed cells. Most interestingly, however, an increase in the basal \([\text{Ca}^{2+}]_i\) was much lower in cells treated with the CA/1,25D3 combination than in those incubated with 100 nM 1,25D3 (Fig. 7, A and B), although the extent of differentiation was comparable (Fig. 7, C and D).

**DISCUSSION**

The dramatic enhancing effect of CA on the 1,25D3-induced monocytic differentiation of HL60-G cells has recently been reported (19), and here we present studies of the mechanistic basis of this potentiation. Our results, taken together with previous studies (39, 50), show that modulation of the cellular redox state appears to enhance monocytic differentiation. The cumulative data indicate that many structurally distinct antioxidants, such as polyphenols, carotenoids, vitamin E, ascorbate, and lipoic acid, all potentiate leukemic cell differentiation induced by various agents, such as 1,25D3 and ATRA (14–17, 50, 51). Thus, the chemical structure of the antioxidant compounds that
that additional mechanisms contribute to its potentiation of differentiation.

Interestingly, exposure of HL60 cells to 1,25D₃ also led to reduction of ROS levels and protection against H₂O₂ stress with even greater capacity than CA. It appears that the effect of 1,25D₃ on the cellular redox status is cell/tissue-dependent, because in breast cancer cells (MCF-7), 1,25D₃ was reported to act as a pro-oxidant (58, 59), whereas in keratinocytes and in neurons, 1,25D₃ protects against various stress stimuli, including H₂O₂ (60, 61). In our study, 1,25D₃ is also an antioxidant and synergizes with CA to markedly reduce the intracellular ROS levels, which may at least in part be related to a synergistic increase in cellular glutathione abundance. Interestingly, although 1,25D₃ alone did not change the basal glutathione content, it greatly potentiated the CA-induced elevation of this peptide concentration. The mechanism of this potentiation is unclear, although it may be related to synergy of the two compounds at the level of glutathione biosynthesis.

The relationship between the extent of differentiation and redox status has not been clearly elucidated, and the available data may seem inconsistent, although cell context and/or experimental conditions must also be taken into consideration, e.g., in HL60 cells induced to differentiate by either DMSO or ATRA, lower levels of ROS and DNA damage and higher levels of GSH were observed, as compared with undifferentiated cells (62). Furthermore, many antioxidants have been shown to induce or enhance differentiation in leukemic cells (Refs. 14–19, 50, and 51 and this study). However, similar differentiation effects were also demonstrated for some agents that promote intracellular ROS formation, e.g., topoisomerase inhibitor β-lapachone (63) or the well-known differentiation inducer butyric acid (64). Granulocytic differentiation of HL60 cells can be induced by the elevation of hydroxyl radicals generated by a Fenton reaction, involving an ADP–Fe²⁺ (or ATP–Fe²⁺) complex and H₂O₂ (65). We demonstrate here that the extent of differentiation in HL60 cells is associated with reducing conditions. Both CA and 1,25D₃ decrease the intracellular ROS levels; various antioxidants potentiate the differentiating effect of 1,25D₃, whereas the pro-oxidant BSO decreases it. Importantly, BSO inhibits not only the differentiation induced by 1,25D₃ but also its potentiation by CA. BSO, a specific inhibitor of γ-glutamylcysteine synthetase that catalyzes the rate-limiting step in glutathione synthesis (33), blocked the increases in glutathione levels by either CA or its combination with 1,25D₃. Thus, the BSO reduction of 1,25D₃-induced differentiation and, particularly, its enhancement by CA suggests that glutathione is involved in both effects, perhaps by protecting the cells from oxidative damage expected to be generated during the oxidative burst that characterizes the function of the matured monocyte (66). Furthermore, changes in GSH levels can lead to the redox-sensitive modulation of transcription regulators, such as AP-1, e.g., via the SH group of its component tripeptide lysine-cysteine-arginine (KCR) (67), as depicted in Fig. 8, or by glutathionylation of diverse proteins that can modify protein function (68).

Activation of AP-1 by 1,25D₃ has been reported previously (23, 69). In the present study, we show that CA/1,25D₃ combinations increase the activation of AP-1 binding by 1,25D₃, whereas CA alone has a minor effect (Fig. 6). Although the mechanism of this activation is still unknown, one possibility is that an up-regulation of the MAPK pathways by the CA/1,25D₃ combination demonstrated here (Fig. 5) also contributes to increased AP-1 activity (Fig. 8). This is supported by previous reports that 1,25D₃ activates ERK in various cells, (34, 35, 70), e.g., Wang and Studzinski (35) have shown that ERK phosphorylation in HL60 cells is much greater during short-term (≤24 h) than long-term (48–96 h) incubations and suggested that the MEK/ERK pathway maintains cell proliferation during the early phase of 1,25D₃-induced monocytic differentiation of HL60 cells but that the


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Carnosic Acid Potentiates the Antioxidant and Prodifferentiation Effects of 1α,25-Dihydroxyvitamin D₃ in Leukemia Cells but Does Not Promote Elevation of Basal Levels of Intracellular Calcium

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